IAEA	International Atomic Energy Agency
ISL	International Standard for Laboratories
ISO	International Organization of Standards
IUPAC	International Union of Pure & Applied Chemistry
NBS	National Bureau of Standards
NIST	National Institute of Standards and Technology
RIVM	National Institute of Public Health and the Environment
UCLA	University of California, Los Angeles
VPDB	Vienna PeeDee Belemnite
VSMOW	Vienna Standard Mean Ocean Water

Application of Gas Chromatography – Combustion – Isotope Ratio Mass Spectrometry to Doping Control

2nd Annual USADA Symposium
on Anti-Doping Science
Los Angeles, California, USA
August 21st – 24th, 2003

Edited By:
Larry D. Bowers, Richard L. Hilderbrand, & Elisha J. Symanski

United States Anti-Doping Agency
Colorado Springs, Colorado, USA

2550 Tenderfoot Hill Street, Suite 200
Colorado Springs, CO 80906
Phone 719.785.2000 • Fax 719.785.2001

NADPH	Nicotinamide-Adenine Dinucleotide Phosphate
NMI	National Measurements Institutes
P2	Pregnanediol
P3	Pregnanetriol
PT	Proficiency Test
PTV	Programmed Temperature Volatilization
RoDH	Retinol DeHydrogenase
RM	Reference material
S	Sulfate
SD	Standard Deviation
SDR	Short-chain dehydrogenase reductase
SI	Systeme International
SPE	Solid Phase Extraction
T	Testosterone
T/E	Ratio of Testosterone/Epi-Testosterone
TBDMS	Tert-Butyl Dimethyl Silyl Group
TC	Target compound
TMIS	Trimethyl iodo Silene
TMP	Total Measurement Process
TMS	Trimethyl Silyl Group
YPD	Yeast extract peptone glucose
VIM	Vocabulary Internationale of Metrology

Miscellaneous	
AGRL	Australian Government Reference Laboratory
AOAC	Association of Analytical Communities
BIPM	International Bureau of Weights & Measures
C-LADA	International Federation of Clinical Chemistry scientific Committee for Laboratory Assessment of Drugs of Abuse

IAEA	International Atomic Energy Agency
ISL	International Standard for Laboratories
ISO	International Organization of Standards
IUPAC	International Union of Pure & Applied Chemistry
NBS	National Bureau of Standards
NIST	National Institute of Standards and Technology
RIVM	National Institute of Public Health and the Environment
UCLA	University of California, Los Angeles
VPDB	Vienna PeeDee Belemnite
VSMOW	Vienna Standard Mean Ocean Water

Application of Gas Chromatography – Combustion – Isotope Ratio Mass Spectrometry to Doping Control

2nd Annual USADA Symposium
on Anti-Doping Science
Los Angeles, California, USA
August 21st – 24th, 2003

Edited By:
Larry D. Bowers, Richard L. Hilderbrand, & Elisha J. Symanski

United States Anti-Doping Agency
Colorado Springs, Colorado, USA

CYP3A4	Cytochrome P450, subfamily IIIA (nifedipine oxidase) polypeptide 4
DHEA	DehydroepiAndrosterone
DHT	Dihydrotestosterone
DTT	Dithiothreitol
E	Etiocholanolone
EA-IRMS	Elemental Analysers - Isotope ratio mass spectrometry
EC	European Commission
Epi-T	Epi-Testosterone
ERC	Endogenous reference compound
G	Glucuronide
GC	Gas chromatography
GC-C-IRMS	Gas chromatography combustion isotope ratio mass spectrometry
GC-MS	Gas chromatography mass spectrometry
hCG	Human Chorionic Gondaotropin
HETP	Heights Equivalent to a Theoretical Plate
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HSD	Hydroxysteroid Dehydrogenases
IM	Intramuscular
IRMS	Isotope ratio mass spectrometry
ISODAT NT	Isotope data systems software
ISOSTER	Project: Determination of the origin of hormones in cattle
LC-MS	Liquid chromatography - mass spectrometry
MS	Mass Spectrometry
MSD	Mass Selective Detector
MSTFA	N-Methyl-N-trimethylsilyltrifluoroacetamide
NAD	Nicotinamide-Adenine Dinucleotide

Preface

The United States Anti-Doping Agency (USADA) was formed in March, 2000 and assumed formal responsibilities for the testing of Olympic, Paralympic, and Pan American athletes; adjudication of doping cases; education; and research in October, 2000. Since its inception, USADA has made a commitment to contribut to the resolution of scientific issues of concern to the global anti-doping community. In reviewing the contributions that USADA might make to the anti-doping movement, there was a clear need for an annual meeting to bring together anti-doping program administrators, laboratory directors, pharmaceutical industry scientists, and academic scientists with expertise in a specific area of interest to the anti-doping community. This meeting could foster improvements in the system and enhance communication and understanding of the issues in doping control for various stakeholders.

USADA's first Annual Symposium on Anti-Doping Science, held in Atlanta, GA in October, 2002, addressed the technology of, testing for, and detection of Oxygen Transport Enhancing Agents and Methods. Eighty-three scientists, doping program administrators, and medical personnel from various international federations (IFs) met and discussed how to eliminate the use of oxygen transport enhancement. As a result of the meeting, critical research needs were identified. These needs have been addressed by both USADA and the World Anti-Doping Agency (WADA) through their respective research grants programs. It is our hope that as a result of this cooperative effort, tests for the oxygen transport enhancing compounds and methods will be part of the routine testing armamentarium of the WADA-accredited laboratories in the near future.

This Second Annual USADA Symposium addressed another difficult issue for anti-doping science -- the detection of anabolic steroids that are normally found in the body. The initial approach to detection of abuse of testosterone (T) was the T/epi-T ratio, which has served the anti-doping movement well for a number of years, but which is prone to false negative results. The purpose of this meeting is to discuss the application of Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) to Doping Analysis. While great progress has been made, particularly in the past five years, this potentially valuable technique has not been widely implemented. There are several reasons for this, but it is clear that definition of the remaining issues and identification of key areas that

need to be resolved scientifically will play a critical role in wider implementation of the technique.

USADA would like to express its thanks to Dr. Don Catlin, a leader in the use of GC-C-IRMS, and to University of California, Los Angeles (UCLA) for their assistance in the planning of the Symposium.

Acronym List

Sport Related		
Acronym	**Definition**	**Description**
CAS	Court of Arbitration for Sport	Court established to hear cases involving sport
IF	International Federation	Organization responsible for the worldwide governance of a particular sport
IOC	International Olympic Committee	
NADO	National Anti-Doping Organization	Organization responsible for the drug testing of elite athletes in a particular country
NFL	National Football League	Organization responsible for the sport of football I the U.S.
UCI	Union Cycliste Internationale	International Federation for Cycling
USADA	United States Anti-Doping Agency	Organization responsible for the drug testing of Olympic, Paralympic, and Pan-American elite athletes throughout the United States
WADA	World Anti-Doping Agency	Organization responsible for the drug testing of elite athletes throughout the world

Scientific and Medical	
17βHSD	17 β hydroxysteroid dehydrogenase
3βHSD	3β–hydroxysteroid dehydrogenase
3-PGA	3-Phosphoglyceric Acid
A	Androsterone
Adiol	Androstanediol
AED	Androstenedione
AKR	Aldo-keto reductase
CITAC	Co-Operation on International Traceability in Analytical Chemistry

CYP3A4	Cytochrome P450, subfamily IIIA (nifedipine oxidase) polypeptide 4
DHEA	DehydroepiAndrosterone
DHT	Dihydrotestosterone
DTT	Dithiothreitol
E	Etiocholanolone
EA-IRMS	Elemental Analysers - Isotope ratio mass spectrometry
EC	European Commission
Epi-T	Epi-Testosterone
ERC	Endogenous reference compound
G	Glucuronide
GC	Gas chromatography
GC-C-IRMS	Gas chromatography combustion isotope ratio mass spectrometry
GC-MS	Gas chromatography mass spectrometry
hCG	Human Chorionic Gondaotropin
HETP	Heights Equivalent to a Theoretical Plate
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HSD	Hydroxysteroid Dehydrogenases
IM	Intramuscular
IRMS	Isotope ratio mass spectrometry
ISODAT NT	Isotope data systems software
ISOSTER	Project: Determination of the origin of hormones in cattle
LC-MS	Liquid chromatography - mass spectrometry
MS	Mass Spectrometry
MSD	Mass Selective Detector
MSTFA	N-Methyl-N-trimethylsilyltrifluoroacetamide
NAD	Nicotinamide-Adenine Dinucleotide

Preface

The United States Anti-Doping Agency (USADA) was formed in March, 2000 and assumed formal responsibilities for the testing of Olympic, Paralympic, and Pan American athletes; adjudication of doping cases; education; and research in October, 2000. Since its inception, USADA has made a commitment to contribut to the resolution of scientific issues of concern to the global anti-doping community. In reviewing the contributions that USADA might make to the anti-doping movement, there was a clear need for an annual meeting to bring together anti-doping program administrators, laboratory directors, pharmaceutical industry scientists, and academic scientists with expertise in a specific area of interest to the anti-doping community. This meeting could foster improvements in the system and enhance communication and understanding of the issues in doping control for various stakeholders.

USADA's first Annual Symposium on Anti-Doping Science, held in Atlanta, GA in October, 2002, addressed the technology of, testing for, and detection of Oxygen Transport Enhancing Agents and Methods. Eighty-three scientists, doping program administrators, and medical personnel from various international federations (IFs) met and discussed how to eliminate the use of oxygen transport enhancement. As a result of the meeting, critical research needs were identified. These needs have been addressed by both USADA and the World Anti-Doping Agency (WADA) through their respective research grants programs. It is our hope that as a result of this cooperative effort, tests for the oxygen transport enhancing compounds and methods will be part of the routine testing armamentarium of the WADA-accredited laboratories in the near future.

This Second Annual USADA Symposium addressed another difficult issue for anti-doping science – the detection of anabolic steroids that are normally found in the body. The initial approach to detection of abuse of testosterone (T) was the T/epi-T ratio, which has served the anti-doping movement well for a number of years, but which is prone to false negative results. The purpose of this meeting is to discuss the application of Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) to Doping Analysis. While great progress has been made, particularly in the past five years, this potentially valuable technique has not been widely implemented. There are several reasons for this, but it is clear that definition of the remaining issues and identification of key areas that

- The Certificate of Analysis or Laboratory Report should include the following
  - Names of target compounds
  - Name(s) of the ERCs
  - $\delta^{13}C$ values of the target compound(s) and the ERC
  - Difference between $\delta^{13}C$ values of the target compound(s) and the ERC
  - Any other information used to determine that the sample was positive (e.g., reference ranges for the individual target compounds)
  - Any opinions should be clearly labeled as such
- Delta values are important and should be in the report
  - Reporting of atom % excess ^{13}C in parts per million may be a more appropriate means of reporting the data. This figure can be derived, for example, from the difference of $\delta^{13}C$ values between the target compound and the ERC.
- The WADA International Standard for Laboratories and Technical Document for Laboratory Documentation Packages apply, although there are specific reporting issues that should be addressed for GC-C-IRMS analysis. The amount of documentation should allow a third party expert to confirm the conclusions derived from the data.
  - Due to the nature of the data, chromatograms should not be required
  - Some measure of peak shape and integrity should be reported
  - Standards for performance verification of the system need to be developed
  - As a system measuring a "quantity," multiple replicates should be analyzed/injected

Table of Contents

Summary of Discussions:

Harmonization of Reporting

- The Certificate of Analysis or Laboratory Report should include the following
  - Names of target compounds
  - Name(s) of the ERCs
  - $\delta^{13}C$ values of the target compound(s) and the ERC
  - Difference between $\delta^{13}C$ values of the target compound(s) and the ERC
  - Any other information used to determine that the sample was positive (e.g., reference ranges for the individual target compounds)
  - Any opinions should be clearly labeled as such
- Delta values are important and should be in the report
  - Reporting of atom % excess ^{13}C in parts per million may be a more appropriate means of reporting the data. This figure can be derived, for example, from the difference of $\delta^{13}C$ values between the target compound and the ERC.
- The WADA International Standard for Laboratories and Technical Document for Laboratory Documentation Packages apply, although there are specific reporting issues that should be addressed for GC-C-IRMS analysis. The amount of documentation should allow a third party expert to confirm the conclusions derived from the data.
  - Due to the nature of the data, chromatograms should not be required
  - Some measure of peak shape and integrity should be reported
  - Standards for performance verification of the system need to be developed
  - As a system measuring a "quantity," multiple replicates should be analyzed/injected

Table of Contents

- Reference materials should be developed and used as internal and external standards in order to evaluate the performance of the system (sample preparation, GC separation, combustion chamber, etc.) and to increase uniformity of between laboratory results
- The use of different target compounds and different ERCs will result in differences between laboratories.
- A WADA-certified batch of characterized acetic anhydride should be made available to all laboratories
- A study comparing **A/E** vs diol test should be completed to assess sensitivity and specificity

Introduction: Application of Gas Chromatography – Combustion - Isotope Ratio Mass Spectrometry to Doping Control- Defining the Issues

Larry D. Bowers, Ph.D.
Senior Managing Director
USADA

The history of the application of isotopic analysis to doping control began with the 1990 abstract by Southan and his colleagues (1) reporting that there was a difference in ^{13}C content between **T** produced in the gonads and pharmaceutical **T**. Both instrumental and sample preparation limitations slowed the development of a routine method with sufficient sensitivity to detect nanogram quantities of steroid and sufficient specificity to detect small differences in ^{13}C content. Michel Becchi and his colleagues (2) reported a GC-C-IRMS method applicable to urine samples in 1994. In the intervening years, several groups, including those of Michel Becchi (3,4), Cedric Shackleton (5,6), Willi Schänzer (7,8,11), Don Catlin (9,12-14) and others (10,15,16), all reported significant steps forward in the measurement of isotopically-enriched steroids using GC-C-IRMS. From this progress, four approaches to GC-C-IRMS analysis are emerging – those using underivatized steroid versus those using a derivatization of the steroid prior to GC analysis and those measuring Androsterone (**A**) and etiocholanolone (**E**) versus those measuring androstanediols (Adiols). Each of these approaches has advantages and disadvantages, and discussion of these strengths and weaknesses will be a major theme of the meeting. In addition, various biological and physiological issues remain to be resolved. For example, several groups have reported a difference in delta values between 5α-Adiol and 5β-Adiol, but the biochemical explanation of this phenomenon is not clear. Shackleton et al (5) observed that diet plays a significant role in establishing the absolute value of the $^{13}C/^{12}C$ ratio. Finally, harmonization of reporting among the laboratories will be an important step toward wider use of GC-C-IRMS.

Another important step in application of GC-C-IRMS testing is its recognition in the anti-doping rules in sport. In 2000, the anti-doping rules of sport began to mention IRMS. For example, the Federation Internationale de Natation (FINA) rules (17) state:

> "In the case of a T/E greater than 6, and **in the absence of an isotope ratio measurement analysis** (*emphasis added*) establishing the presence of exogenous **T**, or its metabolites, or establishing that the **T** analyzed is endogenous, it is mandatory that the relevant medical authority conducts an investigation before the sample is declared positive."

The International Association of Athletics Federations (IAAF) rules (18) state:

> "Evidence from ... isotopic ratio measurements may be used to draw **definite conclusions** *(emphasis added)*."

The Union Cycliste Internationale (UCI) has included the following statement in their rules (19):

> "The Antidoping Commission can have any sample or part of a sample analyzed by a Gas Chromatography/Combustion/ Isotope Ratio Mass Spectrometry in any laboratory which has this apparatus... **If this analysis shows an exogenous application** (*emphasis added*), the rider will be considered positive. In other cases, the Antidoping Commission can ask for further tests..."

And finally in the 2003 International Olympic Committee (IOC)/WADA Prohibited List Explanatory Notes (20), the following footnote is found:

> "6. In prohibited methods, attention is drawn to the fact that Isotope Ratio Mass Spectrometry (IRMS) is considered the preferred method of detecting doping with endogenous androgenic anabolic steroids. It is therefore recommended that IRMS analysis may be applied to any urine that the Laboratory considers has atypical features in the steroid profile."

While this indicates the commitment of these organizations to be on the cutting edge of anti-doping science, the application of GC-C-IRMS to doping control needs thoughtful scientific evaluation.

- Are there doping agents or techniques that could increase **T** concentrations while maintaining a natural $^{13}C/^{12}C$ ratio?
- Are GC-C-IRMS measurements always definitive for an exogenous administration of **T** or its precursors?
- Does GC-C-IRMS replace some of the other analytical tools for doping analysis or does it complement them?

Summary of Discussions:

Harmonization of Inter-laboratory Results

- Reference materials should be developed and used as internal and external standards in order to evaluate the performance of the system (sample preparation, GC separation, combustion chamber, etc.) and to increase uniformity of between laboratory results
- The use of different target compounds and different ERCs will result in differences between laboratories.
- A WADA-certified batch of characterized acetic anhydride should be made available to all laboratories
- A study comparing A/E vs diol test should be completed to assess sensitivity and specificity

Introduction: Application of Gas Chromatography – Combustion - Isotope Ratio Mass Spectrometry to Doping Control- Defining the Issues

Larry D. Bowers, Ph.D.
Senior Managing Director
USADA

The history of the application of isotopic analysis to doping control began with the 1990 abstract by Southan and his colleagues (1) reporting that there was a difference in ^{13}C content between T produced in the gonads and pharmaceutical T. Both instrumental and sample preparation limitations slowed the development of a routine method with sufficient sensitivity to detect nanogram quantities of steroid and sufficient specificity to detect small differences in ^{13}C content. Michel Becchi and his colleagues (2) reported a GC-C-IRMS method applicable to urine samples in 1994. In the intervening years, several groups, including those of Michel Becchi (3,4), Cedric Shackleton (5,6), Willi Schänzer (7,8,11), Don Catlin (9,12-14) and others (10,15,16), all reported significant steps forward in the measurement of isotopically-enriched steroids using GC-C-IRMS. From this progress, four approaches to GC-C-IRMS analysis are emerging – those using underivatized steroid versus those using a derivatization of the steroid prior to GC analysis and those measuring Androsterone (A) and etiocholanolone (E) versus those measuring androstanediols (Adiols). Each of these approaches has advantages and disadvantages, and discussion of these strengths and weaknesses will be a major theme of the meeting. In addition, various biological and physiological issues remain to be resolved. For example, several groups have reported a difference in delta values between 5α-Adiol and 5β-Adiol, but the biochemical explanation of this phenomenon is not clear. Shackleton et al (5) observed that diet plays a significant role in establishing the absolute value of the $^{13}C/^{12}C$ ratio. Finally, harmonization of reporting among the laboratories will be an important step toward wider use of GC-C-IRMS.

Another important step in application of GC-C-IRMS testing is its recognition in the anti-doping rules in sport. In 2000, the anti-doping rules of sport began to mention IRMS. For example, the Federation Internationale de Natation (FINA) rules (17) state:

7. Assuming that points 1-6 are properly addressed, getting the same $\delta^{13}C$ (and δD) in different labs requires very close attention to the (often arcane) details of reference gases, primary and secondary standards. This, however, is not the purview of the manufacturer.

REFERENCES

1. Assonov S.S. and C.A.M. Brenninkmeijer, 2003, **On the ^{17}O correction for CO_2 mass spectrometer isotopic analysis.** *Rapid Communications in Mass Spectrometry*, 17, 1007-1016

The rules of sport have other important definitions that impact doping control. First, an adverse analytical finding in the WADA Code requires only the detection of a prohibited substance or its metabolites or markers in the sample from the athlete. Second, the principle of strict liability applies meaning that the athlete is held accountable for whatever is found in his or her body or body fluids. It does not matter what the intent was. It does not matter how it got there. Third, the athlete's rights include the right to a hearing before an arbitration panel, such as the Court of Arbitration for Sport (CAS). Finally, the burden of proof for the prosecution in such an arbitration hearing according to the WADA Code is "the comfortable satisfaction of the hearing body bearing in mind the seriousness of the allegation which is being made." This is higher than a preponderance of evidence (as would be in effect in a US civil proceeding) but lower than beyond a reasonable doubt (as in a criminal proceeding). As we discuss the technique of GC-C-IRMS, our focus should be on the scientific reliability of the GC-C-IRMS technique in establishing, or ruling out, the administration of a prohibited substance.

REFERENCES

1. Southan G, Mallet A, Jumeau J, Craig S, Poojara N, et al. Programme and Abstracts of the 2nd International Symposium of Applied Mass Spectrometry in the Health Sciences, Barcelona, Spain, 1990, p 306.
2. Becchi M, Aguilera R, Farizon Y, Flament MM, Casabianca H, James P. Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of T in sport. *Rapid Commun Mass Spectrom* 1994; **8**: 304-8.
3. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, et al. Improved method of detection of T abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. *J Mass Spectrom* 1996; **21**: 169-76.
4. Aguilera R, Becchi M, Grenot C, Casabianca H, Hatton CK. Detection of T misuse: comparison of two chromatographic sample preparation methods for gas chromatographic-combustion/isotope ratio mass spectrometric analysis. *J Chromatogr B Biomed Appl* 1996; **687**:43-53.
5. Shackleton CH, Phillips A, Chang T, Li Y. Confirming T administration by isotope ratio mass spectrometric analysis of urinary Adiols. *Steroids* 1997; **62**: 379-87.

6. Shackleton CH, Roitman E, Phillips A, Chang T. Androstanediol and 5-androstenediol profiling for detecting exogenously administered dihydrotestosterone, epi-testosterone, and dehydroepiAndrosterone: potential use in gas chromatography isotope ratio mass spectrometry. *Steroids* 1997; **62**: 665-73.
7. Horning S, Geyer H, Machnik M, Schänzer W, Hilkert A, and Oeßelmann J. Detection of exogenous steroids by $^{13}C/^{12}C$ analysis. In *Recent Advances in Doping Analysis (4) – Proceedings of the 14th Cologne Workshop on Dope Analysis 1996*, W Schänzer , H Geyer, A Gotzmann, and U Mareck-Engelke (Eds). Sport and Buch Strauß, Köln, 1997. pp 275-83.
8. Horning S, Geyer H, Gotzmann A, Schänzer W. Detection of exogenous steroids by $^{13}C/^{12}C$ analysis. In *Recent Advances in Doping Analysis (5) – Proceedings of the 15th Cologne Workshop on Dope Analysis 1997*, W Schänzer , H Geyer, A Gotzmann, and U Mareck-Engelke (Eds). Sport and Buch Strauß, Köln, 1998. pp 135-48.
9. Aguilera R, Catlin DH, Becchi M, Phillips A, Wang C, et al. Screening urine for exogenous T by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols. *J Chromatogr B Biomed Sci Appl* 1999; **727**: 95-105.
10. Ueki M, Okano M. Analysis of exogenous dehydroepiAndrosterone excretion in urine by gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* 1999; **13**: 2237-43.
11. Flenker U, Horning S, Nolteernsting E, Geyer H, Schänzer W. Measurement of $^{12}C/^{13}C$-ratios to Confirm Misuse of Endogenous Steroids. In *Recent Advances in Doping Analysis (6) – Proceedings of the 16th Cologne Workshop on Dope Analysis 1998*, W Schänzer , H Geyer, A Gotzmann, and U Mareck-Engelke (Eds). Sport and Buch Strauß, Köln, 1999. pp 243-56.
12. Aguilera R, Chapman TE, Catlin DH. A rapid screening assay for measuring urinary A and E $\delta^{13}C$ (‰) values by gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* 2000; **14**: 2294-9.
13. Bourgogne E, Herrou V, Mathurin J-C, Becchi M, de Ceaurriz J. Detection of exogenous intake of natural corticosteroids by gas chromatography/combustion/isotope ratio mass spectrometry : application to misuse in sport. Rapid Commun Mass Spectrom 2000; **14**: 2343-7.

GC-C-IRMS: getting the right numbers

Charles Douthitt
ThermoFinnigan

The isotope ratio mass spectrometer (IRMS) is designed to precisely and accurately measure the difference in isotopic compositions between a sample and a reference gas. Considerations significant to achieving this goal in the practice of GC-IRMS include:

CAPILLARY GC: *the best possible chromatographic resolution is imperative*

1. Compounds undergo isotope fractionation in a capillary GC column, so that the front of a peak is generally enriched in ^{13}C relative to the tail. It is not possible to perform peak deconvolution on GC-IRMS data, so the most accurate data requires the best possible separation of peaks
2. In order to ensure the most consistent GC performance, the new ThermoFinnigan ISODAT NT software includes full control over GC methods, injectors and auto samplers

GC INTERFACE: *chromatographic resolution can only get worse, it can't be improved*

3. The major design goal for the interface between the capillary GC and the IRMS was to do nothing that could degrade chromatographic resolution (e.g., no cold spots, no T connections, the reactors are small bore and are not packed)

SOFTWARE: *data collection and data correction*

4. The software must properly and consistently perform determination of baselines, peak integration and time shift corrections (required because of the isotope fractionation discussed in #1). The new ISODAT NT software includes significant enhancements to peak integration and baseline correction algorithms
5. Proper correction for the contribution of $^{12}C^{17}O^{16}O$ to mass 45. The new ISODAT NT software incorporates the newest and most powerful methods for performing this correction[1].

IRMS PERFORMANCE: *linearity*

6. While sensitivity is an important consideration (how small a sample can one analyze?), an often overlooked but equally important consideration is linearity. Linearity should be a specification, and it should be small and consistent and it should be possible to correct raw data to eliminate any effect of varying sample size

STANDARDIZATION: *getting onto the V-PDB scale*

7. Assuming that points 1-6 are properly addressed, getting the same $\delta^{13}C$ (and δD) in different labs requires very close attention to the (often arcane) details of reference gases, primary and secondary standards. This, however, is not the purview of the manufacturer.

REFERENCES

1. Assonov S.S. and C.A.M. Brenninkmeijer, 2003, **On the ^{17}O correction for CO_2 mass spectrometer isotopic analysis.** *Rapid Communications in Mass Spectrometry*, 17, 1007-1016

The rules of sport have other important definitions that impact doping control. First, an adverse analytical finding in the WADA Code requires only the detection of a prohibited substance or its metabolites or markers in the sample from the athlete. Second, the principle of strict liability applies meaning that the athlete is held accountable for whatever is found in his or her body or body fluids. It does not matter what the intent was. It does not matter how it got there. Third, the athlete's rights include the right to a hearing before an arbitration panel, such as the Court of Arbitration for Sport (CAS). Finally, the burden of proof for the prosecution in such an arbitration hearing according to the WADA Code is "the comfortable satisfaction of the hearing body bearing in mind the seriousness of the allegation which is being made." This is higher than a preponderance of evidence (as would be in effect in a US civil proceeding) but lower than beyond a reasonable doubt (as in a criminal proceeding). As we discuss the technique of GC-C-IRMS, our focus should be on the scientific reliability of the GC-C-IRMS technique in establishing, or ruling out, the administration of a prohibited substance.

REFERENCES

1. Southan G, Mallet A, Jumeau J, Craig S, Poojara N, et al. Programme and Abstracts of the 2nd International Symposium of Applied Mass Spectrometry in the Health Sciences, Barcelona, Spain, 1990, p 306.
2. Becchi M, Aguilera R, Farizon Y, Flament MM, Casabianca H, James P. Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of **T** in sport. *Rapid Commun Mass Spectrom* 1994; **8**: 304-8.
3. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, et al. Improved method of detection of **T** abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. *J Mass Spectrom* 1996; **21**: 169-76.
4. Aguilera R, Becchi M, Grenot C, Casabianca H, Hatton CK. Detection of **T** misuse: comparison of two chromatographic sample preparation methods for gas chromatographic-combustion/isotope ratio mass spectrometric analysis. *J Chromatogr B Biomed Appl* 1996; **687**:43-53.
5. Shackleton CH, Phillips A, Chang T, Li Y. Confirming T administration by isotope ratio mass spectrometric analysis of urinary Adiols. *Steroids* 1997; **62**: 379-87.

AGUILERA
How much difference is there in between one pulse and another one, in delta values?

PHILLIPS
It depends on what the matrix background is that you drop it in on. But the system will still do background subtraction on the reference peak (prior to the reference peak).

14. Aguilera R, Chapman TE, Starcevic B, Hatton CK, Catlin DH. Performance characteristics of a carbon isotope ratio method for detecting doping with T based on urine diols: controls and athletes with elevated T/epi-T ratios. *Clin Chem* 2001; **47**: 292-300.
15. Aguilera R, Hatton CK, Catlin DH. Detection of epi-T doping by isotope ratio mass spectrometry. *Clin Chem* 2002; **48**: 629-36.
16. Ayotte C, Goudreault D, Lajeunesse A, Cléroux M, Richard Y, et al. GC-C-IRMS and GC/MS in "Natural" Steroids Testing. In *Recent Advances in Doping Analysis (9) – Proceedings of the 19th Cologne Workshop on Dope Analysis 2001*, W Schänzer , H Geyer, A Gotzmann, and U Mareck-Engelke (Eds). Sport and Buch Strauß, Köln, 2002. pp 133-43.
17. Addendum to FINA Handbook 2002-2005 (December 2002). Appendix A. Section I.C.1., p. 18
18. IAAF Procedural Guidelines for Doping Control, 2000 edition. Schedule 1, Part I(a)(I), p 18.
19. UCI Prohibited Classes of Substances and Prohibited Methods (1st April 2000), Section I.C., p 3.
20. IOC/WADA List of Prohibited Substances

Attendee List

Dr. Rodrigo Aguilera
Researcher
Pasarow Mass Spectrometry Laboratory, UCLA
United States

Mr. Brian Ahrens
Researcher & Certifying Scientist
UCLA Olympic Analytical Laboratory
United States

Dr. Tongtavuch Anukarahanonta
Head of the IOC/WADA Laboratory
National Doping Control Centre, Mahidol University
Thailand

Prof. Francisco Radler Aquino Neto
Head of the IOC/WADA Laboratory
LABDOP-Ladetec/IQ-UFRJ, Centro de Technologia
Brazil

Dr. Richard Auchus
Assistant Professor of Endocrinology and Metabolism
University of Texas
United States

Mr. Michel Becchi
Research Scientist
Centre National de la Recherche Scientifique
France

Mr. William Bock
USADA Counsel
KROGER, GARDIS & REGAS, L.L.P.
United States

Ms. Kate Borg
Board of Directors
USADA
United States

Prof. Dr. Francesco Botré
Head of the IOC/WADA Laboratory
Federazione Medico Sportiva Italiana, Laboratorio Antidoping
Italy

Derivatized samples have shown to improve the chromatographic separation, but the choice of materials is reduced due to the combustion of the agent attacking the furnace catalyst, also they add extra Carbon atoms that will affect the delta value. Corrections can be made for the addition of the extra Carbons.

To improve the harmonization of the laboratories they need to be analyzing the same test mixture and obtaining the correct values before analyzing the samples. So a Test Mixture needs to be standardized and available to the laboratories.

At present they report the measured delta value of the steroid metabolites, it might be more accurate reporting a ratio values between the different naturally occurring metabolites, and this could eliminate differences between integration logarithms, and also could eliminate the need to correct for the extra carbons added during derivatization.
As long as all the laboratories follow the same protocol then all results should be traceable. Occasional round robin analysis of sets of samples will monitor the laboratories measurements.

Question & Answer Discussion

PHILLIPS
The main thing being that you make sure you put it in the right compound in a position, so it is not going to co-elute with something. I think Prof. Ueki has been adding internal standards with quite a lot of his samples. So straight away, as well as, running standards before you start any samples, you can look down your results and make sure that all your internal standards are staying at the same value.

AGUILERA
You are using the injection of the CO_2 peak still in "backflush off" or "backflush on" in the new software to do the calculation of the value? You better remember if you inject a CO_2 during the "backflush off" and after, you switch to "backflush on". Are you doing the analysis and after you inject again to CO_2 and "backflush off"?

PHILLIPS
That is the standard way we would do analysis but you can actually put the CO_2 pulse the same time that the GC flow is going in.

AGUILERA
How much difference is there in between one pulse and another one, in delta values?

PHILLIPS
It depends on what the matrix background is that you drop it in on. But the system will still do background subtraction on the reference peak (prior to the reference peak).

14. Aguilera R, Chapman TE, Starcevic B, Hatton CK, Catlin DH. Performance characteristics of a carbon isotope ratio method for detecting doping with T based on urine diols: controls and athletes with elevated T/epi-T ratios. *Clin Chem* 2001; **47**: 292-300.
15. Aguilera R, Hatton CK, Catlin DH. Detection of epi-T doping by isotope ratio mass spectrometry. *Clin Chem* 2002; **48**: 629-36.
16. Ayotte C, Goudreault D, Lajeunesse A, Cléroux M, Richard Y, et al. GC-C-IRMS and GC/MS in "Natural" Steroids Testing. In *Recent Advances in Doping Analysis (9) – Proceedings of the 19th Cologne Workshop on Dope Analysis 2001*, W Schänzer , H Geyer, A Gotzmann, and U Mareck-Engelke (Eds). Sport and Buch Strauß, Köln, 2002. pp 133-43.
17. Addendum to FINA Handbook 2002-2005 (December 2002). Appendix A. Section I.C.1., p. 18
18. IAAF Procedural Guidelines for Doping Control, 2000 edition. Schedule 1, Part I(a)(I), p 18.
19. UCI Prohibited Classes of Substances and Prohibited Methods (1st April 2000), Section I.C., p 3.
20. IOC/WADA List of Prohibited Substances

The original developments for stable isotopes techniques were mostly dedicated to geochemistry applications. With the evolution of the techniques the scientists working in the field of Cosmo chemistry, hydrology, environment, doping control, food control (adulteration), medical research, pharmaceutical industry and now in forensic science as well have adopted stable isotope analysis as an essential tool in their laboratories.

Misuse of T has become a classical doping case in sport. The IOC and all sport IFs ban its use. Considering the natural variability of T concentration in individuals coming from different origins, measuring concentrations of T metabolites in urines was not specific enough and a method to differentiate natural and synthetic T was needed, so GC IRMS was introduced.

At present sports laboratories are analyzing steroid metabolites by GC IRMS and compiling huge libraries of results on gender, origin and diets, but the questions lies can the same sample give the same result if measured in another laboratory on a different instrument?

The answer to this question is yes.

However the laboratories will have to harmonize their approach to the analysis. This means that every analysis has to be traceable to the Instrument calibration material, which in turn must be traceable to the international standard VPDB for CO2 measurements.

At present there are many commercial standards traceable to VPDB, that can be obtained from NIST, EIL and IAEA but none of them can be analyzed by GC. This leaves some laboratories that do not have other isotope techniques, vulnerable at not being able to manufacture calibrated secondary standards. It is important that certain criteria are achievable to give the correct values before attempting to measure any sample by GC IRMS. They are combustion, water removal, sample peak integration and the reference gas that has been calibrated with respect to an international standard or against the secondary standards. The correct delta values of the GC test material must be obtained before analyzing any samples, the test material should be analyzed at frequent intervals between sample analyses.

Sample preparation is extremely important and the cleaner the sample then the more accurate and reproducible the results will be. Unlike other forms of GC IMS, IRMS does not have high resolution or MS/MS capabilities in being selective. Any impurities around the sample peaks that enter the combustion furnace will be combusted to CO_2 and enter the IRMS.

Dr. Larry Bowers
Senior Managing Director
USADA
United States

Prof. James Thomas Brenna
Professor
Cornell University
United States

Mr. Stephane Carando
Supervisor in IOC/WADA Laboratory
National Doping Laboratory, CREPS
France

Prof. Dr. Don Catlin
Head of the IOC/WADA Laboratory
UCLA Olympic Analytical Laboratory
United States

Dr. Richard Cohen
Board of Directors
USADA
United States

Mrs. Carol Congdon
Executive Administrator
USADA
United States

Prof. David Cowan
Head of the IOC/WADA Laboratory
Drug Control Centre, King's College London
United Kingdom

Prof. Dr. FT. Frans Delbeke
Head of the IOC/WADA Laboratory
Doping Control Laboratory, Ghent University
Belgium

Dr. Bryan Finkle
Consultant, Forensic Toxicologist
National Football League (NFL)
Unites States

Dr. Jean Fourcroy
Board of Directors
USADA
United States

Dr. Mats Garle
Head of the IOC/WADA Laboratory
Doping Control Laboratory, Huddinge University Hospital
Sweden

Dr. Costas Georgakopoulos
Head of the IOC/WADA Laboratory
Olympic Athletics Center of Athens
Greece

Mr. Joachim Grosse
German Sports University
Germany

Dr. Ralph Hale
Board of Directors
USADA
United States

Dr. Caroline Hatton
Consultant
UCLA Olympic Analytical Laboratory
United States

Prof. Peter Hemmersbach
Head of the IOC/WADA Laboratory
Hormone Laboratory, Aker University Hospital
Norway

Dr. Richard Hilderbrand
Science Director
USADA
United States

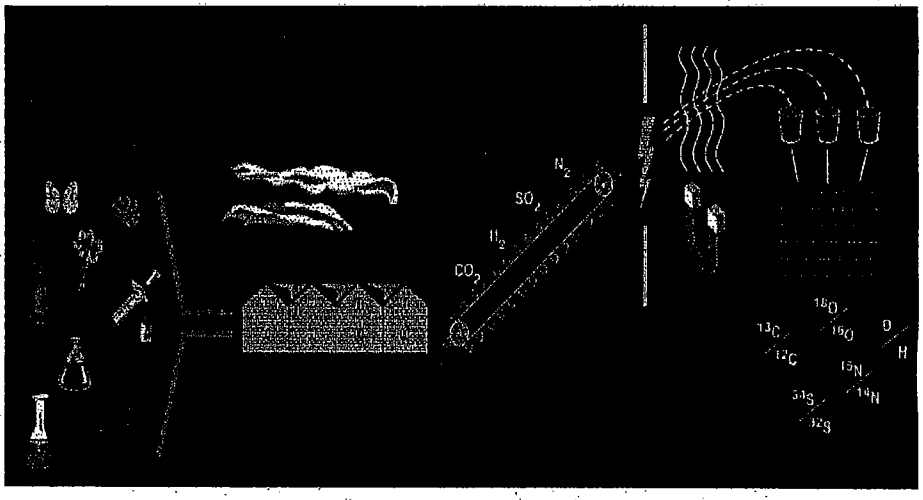
Prof. Edward Houghton
Chief Scientist, Director
Horseracing Forensic Laboratory
United Kingdom

Dr. Marilyn Huestis
Acting Chief, Chemistry and Drug Metabolism Section
National Institute on Drug Abuse
United States

Inter Laboratory Harmonization

Andy Phillips
GV Instruments

The power of stable isotopes techniques lies in the fact that Isotope Ratio Mass Spectrometry (IRMS) can provide information relating to the origin of the analyzed compounds. That can be used to differentiate natural/artificial compounds, endogenous/exogenous compounds, to determine metabolic pathways, geographic origins…The stable isotope mass spectrometer is a gas source instrument which can analyze very precisely very small variations on the isotopic composition of a limited number of gas species. Typically, $^{13}C/^{12}C$ is measured as CO_2, $^{15}N/^{14}N$ is measured as N_2, D/H is measured as H_2, $^{18}O/^{16}O$ can be measured as CO_2, CO or O_2; $^{34}S/^{32}S$ is measured as SO_2 or SO. Although in the past, the key to stable isotope determinations was the performance of the mass spectrometer itself, today an essential part of any IRMS system is the inlet system that will automatically transform the sample into gas species, which the IRMS can analyze. Stable isotopes can be used at natural abundance level, looking at the natural variations of isotope ratios during biogeochemical processes, or they can be used with tracer compounds artificially enriched in one of the isotopes.



The original developments for stable isotopes techniques were mostly dedicated to geochemistry applications. With the evolution of the techniques the scientists working in the field of Cosmo chemistry, hydrology, environment, doping control, food control (adulteration), medical research, pharmaceutical industry and now in forensic science as well have adopted stable isotope analysis as an essential tool in their laboratories.

Misuse of T has become a classical doping case in sport. The IOC and all sport IFs ban its use. Considering the natural variability of T concentration in individuals coming from different origins, measuring concentrations of T metabolites in urines was not specific enough and a method to differentiate natural and synthetic T was needed, so GC IRMS was introduced.

At present sports laboratories are analyzing steroid metabolites by GC IRMS and compiling huge libraries of results on gender, origin and diets, but the questions lies can the same sample give the same result if measured in another laboratory on a different instrument?

The answer to this question is yes.

However the laboratories will have to harmonize their approach to the analysis. This means that every analysis has to be traceable to the Instrument calibration material, which in turn must be traceable to the international standard VPDB for CO2 measurements.

At present there are many commercial standards traceable to VPDB, that can be obtained from NIST, EIL and IAEA but none of them can be analyzed by GC. This leaves some laboratories that do not have other isotope techniques, vulnerable at not being able to manufacture calibrated secondary standards. It is important that certain criteria are achievable to give the correct values before attempting to measure any sample by GC IRMS. They are combustion, water removal, sample peak integration and the reference gas that has been calibrated with respect to an international standard or against the secondary standards. The correct delta values of the GC test material must be obtained before analyzing any samples, the test material should be analyzed at frequent intervals between sample analyses.

Sample preparation is extremely important and the cleaner the sample then the more accurate and reproducible the results will be. Unlike other forms of GC IMS, IRMS does not have high resolution or MS/MS capabilities in being selective. Any impurities around the sample peaks that enter the combustion furnace will be combusted to CO_2 and enter the IRMS.

Dr. Larry Bowers
Senior Managing Director
USADA
United States

Prof. James Thomas Brenna
Professor
Cornell University
United States

Mr. Stephane Carando
Supervisor in IOC/WADA Laboratory
National Doping Laboratory, CREPS
France

Prof. Dr. Don Catlin
Head of the IOC/WADA Laboratory
UCLA Olympic Analytical Laboratory
United States

Dr. Richard Cohen
Board of Directors
USADA
United States

Mrs. Carol Congdon
Executive Administrator
USADA
United States

Prof. David Cowan
Head of the IOC/WADA Laboratory
Drug Control Centre, King's College London
United Kingdom

Prof. Dr. FT. Frans Delbeke
Head of the IOC/WADA Laboratory
Doping Control Laboratory, Ghent University
Belgium

Dr. Bryan Finkle
Consultant, Forensic Toxicologist
National Football League (NFL)
Unites States

Dr. Jean Fourcroy
Board of Directors
USADA
United States

my lab. Yes, you have to get the strain though, and my understanding is the industrial partner, like you said, decided this was not going to be economically feasible. So, those strains are probably frozen away somewhere and are probably not going to be used for production of hydrocortisone. To answer your question about, can you make androgens? We make DHEA with yeast all the time, just adding cytochrome B5 to the cocktail they have there, to increase the lyase activity. So, it would be a trivial step to do that.

The difference in the delta values is quite amazing. Do you know if those delta values are also different if you take regular yeast and isolate the ergosterol, their normal product, which could potentially be a source of synthetic androgens from the standard YPD cocktail, what you would get?

BECCHI
Yes, the value would be totally very different but have not measured the ergosterol, for instance. It is possible to do this.

Dr. Rymantas Kazlauskas
Head of the IOC/WADA Laboratory
Australian Government Analytical Laboraories
Australia

Dr. Aishah Latiff
Head of the IOC/WADA Laboratory
Doping Control Centre, Universiti Sains
Malaysia

Prof. Bruno Le Bizec
Head Deputy - Scientific Manager
LABERCA - French Reference Laboratory
France

Mr. Antti Leinonen
Technical Manager in IOC/WADA Laboratory
Doping Control Laboratory, United Laboratories Ltd
Finland

Mrs. Hanne Solveig Lund
Scientist
Hormone Laboratory, Aker University Hospital
Norway

Mr. Terry Madden
Chief Executive Officer
USADA
United States

Prof. Dwight Matthews
Professor & Chair, Chemistry & Medicine
The University of Vermont
United States

Mr. Andy Phillips
Demonstration Laboratory Manager
GV Instruments
United Kingdom

Dr. Angelika Preiss-Weigert
Deputy Head of Instrumental Analysis
Federal Institute for Risk Assessment
Germany

Dr. Doug Rollins
Professor, Pharmacology and Toxicology
Center for Human Toxicology, University of Utah
United States

Mr. Edward Ryan
Sports Medicine Director
U.S. Olympic Committee
United States

Dr. Martial-Olivier Saugy
Head of the IOC/WADA Laboratory
Laboratory for doping analyses, Institut Universitaire de Medecine Legale
Switzerland

Prof. Jordi Segura
Head of the IOC/WADA Laboratory
Institut Municipal Investigació Mèdica
Spain

Mr. Michael Sekera
Scientific Director
UCLA Olympic Analytical Laboratory
United States

Dr. Matthew Slawson
Assistant Director
Center for Human Toxicology, University of Utah
United States

Mrs. Saskia Sterk
Head Section Veterinary Drugs
National Institute of Public Health and the Environment (RIVM)
Netherlands

Dr. Hiraku Suzuki
Chief Manager/Special Chemistry Group
Mitsubishi Kagaku Bio-Clinical Laboratories INC.
Japan

Mrs. Elisha Symanski
Operations Manager
USADA
United States

Dr. Mario Thevis
German Sports University
Germany

Mr. Travis Tygart
Legal Affairs Director
USADA
Unites States

C-IRMS differentiation must be done with attention as this corticosteroid metabolite can have an exogenous origin.

REFERENCES

1. *Self-sufficient biosynthesis of pregnenolone and progesterone in engineered yeast*, C. Duport, R. Spagnoli, E. Degryse, and D. Pompon - Nature Biotechnology 16 (1998), 186-189.
2. *Total biosynthesis of hydrocortisone from a simple carbon source in yeast*, B. Dumas *et al.*-Nature Biotechnology 21 (2003), 143-149.
3. *Detection of exogenous intake of natural corticosteroids by GC-C-IRMS: application to misuse in sport*, E. Bourgogne, V. Herrou, J.-C. Mathurin, M. Becchi and J. de Ceaurriz – Rapid Communications in Mass Spectrometry 14 (2000) 233-2347.

Question & Answer Discussion

BECCHI
I think at the moment it is an economical program, the moment the steroids from plants more economical than the yeast, and they are maybe to improve their methods. Actually in the publication, the rate of production was 10 mcg per ml of culture, so it will depend on the economical programs condition.

BOWERS
Do you have plans to analyze the corticosteroid products from yeast?

BECCHI
No, I am no longer involved in this field.

VERKOUTEREN
I was just interested in this designer yeast too. Can the yeast replicate itself and maintain the same functionality?

BECCHI
I do not know at the moment. You have to ask Dr. Pompons who is the specialist on the yeast.

AUCHUS
I know I use Dr. Pompons' genetically altered yeast, not this strain, but we use his system for expressing some of our enzymes in yeast all the time in

my lab. Yes, you have to get the strain though, and my understanding is the industrial partner, like you said, decided this was not going to be economically feasible. So, those strains are probably frozen away somewhere and are probably not going to be used for production of hydrocortisone. To answer your question about, can you make androgens? We make DHEA with yeast all the time, just adding cytochrome B5 to the cocktail they have there, to increase the lyase activity. So, it would be a trivial step to do that.

The difference in the delta values is quite amazing. Do you know if those delta values are also different if you take regular yeast and isolate the ergosterol, their normal product, which could potentially be a source of synthetic androgens from the standard YPD cocktail, what you would get?

BECCHI
Yes, the value would be totally very different but have not measured the ergosterol, for instance. It is possible to do this.

Dr. Rymantas Kazlauskas
Head of the IOC/WADA Laboratory
Australian Government Analytical Laboraories
Australia

Dr. Aishah Latiff
Head of the IOC/WADA Laboratory
Doping Control Centre, Universiti Sains
Malaysia

Prof. Bruno Le Bizec
Head Deputy - Scientific Manager
LABERCA - French Reference Laboratory
France

Mr. Antti Leinonen
Technical Manager in IOC/WADA Laboratory
Doping Control Laboratory, United Laboratories Ltd
Finland

Mrs. Hanne Solveig Lund
Scientist
Hormone Laboratory, Aker University Hospital
Norway

Mr. Terry Madden
Chief Executive Officer
USADA
United States

Prof. Dwight Matthews
Professor & Chair, Chemistry & Medicine
The University of Vermont
United States

Mr. Andy Phillips
Demonstration Laboratory Manager
GV Instruments
United Kingdom

Dr. Angelika Preiss-Weigert
Deputy Head of Instrumental Analysis
Federal Institute for Risk Assessment
Germany

Dr. Doug Rollins
Professor, Pharmacology and Toxicology
Center for Human Toxicology, University of Utah
United States

Towards new $\delta^{13}C$ signature for steroids?

Michel Becchi
Centre National de la Recherche Scientifique

Gonadal T is made from precursor molecules derived from a wide variety of food materials. In human bodies, the ^{13}C content of T and its metabolites are generally found at about -25‰ for δ ^{13}C values, depending on all the nature of the food eaten and the discrimination of the enzymes involve in the biosynthesis pathways. Synthetic T is made from a plant species, mostly soy, with δ ^{13}C values of about -29‰, allowing differentiation of the human one.

The production of pregnenolone, an important intermediate of steroidal drug synthesis was reported from a simple carbon source (ethanol) by recombinant yeast *Saccharomyces cerevisiae* strains (1).

We studied the influence of carbon source on the resulting pregnenolone production from this engineered yeast. The carbon source came from ethanol with two different ^{13}C values (ethanol from sugar cane and from sugar beet). The results of δ ^{13}C determination were presented in the following Table:

Compounds	^{13}C δ‰
Pregnenolone acetate from SIGMA Carbon source: plant sterols?	- 36.1
Pregnenolone acetate from Yeast Carbon source : Ethanol from sugar beet	- 19.0
Ethanol from sugar beet	- 26.5
Pregnenolone acetate from Yeast Carbon source : Ethanol from sugar cane	- 3.1
Ethanol from sugar cane	- 12.1

There was a strong discrimination of carbon incorporation into the biosynthetic recombinant yeast steroids. The δ ^{13}C was very different from the natural human steroids one.

More recently, hydrocortisone and cortisone were also produced by other recombinant yeast (2). Corticosteroids could easily be studied by GC-C-IRMS as androstan-tri-one derivatives (3), and, as anabolic steroids, the differentiation between synthetic and natural human ones was effective. The choice of 11-hydroxy-etiocholanolone as an ERCs for anabolic steroids GC-

Dr. Makoto Ueki
Head of the IOC/WADA Laboratory
Mitsubishi Kagaku Bio-Clinical Laboratories, Inc.
Japan

Dr. Michael Verkouteren
Research Chemist
National Institute of Standards and Technology
United States

Prof. Christina Wang
Program Director
Harbor-UCLA Medical Center
United States

Mr. Rich Wanninger
Communications & Public Affairs Director
USADA
United States

Ms. Andrea Wickerham
Legal Relations and Policy Director
The National Center for Drug Free Sport
United States

Dr. Prof. Mountian Wu
Head of the IOC/WADA Laboratory
China Doping Control Center
China

Mr. Richard Young
USADA Counsel
Holme, Roberts & Owen, LLP
United States

Dr. Mario Zorzoli
Official Doctor
Union Cycling Internationale (UCI)
Switzerland

Recommendations and Conclusions

- A small expert panel should be convened to consider what specific assay improvements are needed, guidelines should be used, and make recommendations to WADA. These recommendations may include guidelines on assay characteristics and performance criteria that will unify the method(s) that are used in doping control.
  - Build on the foundation provided by the IOC Working Group
  - Develop a Proficiency Testing Round Robin to improve harmonization of reported values

- USADA should fund a 50-subject reference range study in the eleven labs currently reporting GC-C-IRMS data
  - Reference material and/or internal standard must be identified and produced
  - Target substances must be identified and run in all samples
  - Specify internal standard and reporting requirements

- There are ways to beat the GC-C-IRMS test, such as administration of hCG, aromatase inhibitors, or clomiphene.
  - Doping may have occurred, even in the presence of a negative GC-C-IRMS test;
  - A positive GC-C-IRMS does definitively support the administration of pharmaceutical T or T precursors
  - An expert panel needs to provide recommendations on the combination of longitudinal testing and GC-C-IRMS to provide cost-effective testing strategies
  - A GC-C-IRMS may be required in every case of an elevated T/E ratio

- The reporting of differences of $\delta^{13}C$ values (using an endogenous reference compound) as opposed to the ratio of $\delta^{13}C$ values is strongly recommended. There may be additional benefit in considering the absolute $\delta^{13}C$ values compared to the reference ranges of individual target analytes.

- A Reference Material and/or Internal Standard material should be developed to assist the laboratories in achieving more uniform results.

are going to get a lot of false negatives, unless we can get the criteria really well worked out.

COWEN
Have you considered how the approach you are using is going to fit in to International Standard for Laboratories (ISL) of WADA for next year? Dr. Bowers' comments about screening, confirmatory test. How are you going to apply it? Might the ISL need a separate document to deal with GC-C-IRMS? I was thinking about some discrimination between the screening method, and the confirmatory method that we are required to do.

KAZLAUSKAS
If you are using this as a screening and you actually insist the confirmation be something completely different, in which we have to say the diols, rather than be able to repeat this, or have some other procedure. I do not know.

AGUILERA
Do you know why in some cases the distribution is very tight and some populations it is spread out? I do not think about the shift in the average but talking about the distribution for in the same cases you going from –15 to –25 and then other cases you are using a very tight distribution of the population.

KAZLAUSKAS
Yes, I can think of lots of reasons why they might. I mean maybe these athletes travel. A lot of it is diet related and it may be they travel. They may be mixed populations in terms of genetics. We know for example in the T/E, the Asian population is mixed. You have got those who have got very low T/Es, and you got those who have a T/E like Caucasians.

Towards new $\delta^{13}C$ signature for steroids?

Michel Becchi
Centre National de la Recherche Scientifique

Gonadal T is made from precursor molecules derived from a wide variety of food materials. In human bodies, the ^{13}C content of T and its metabolites are generally found at about -25‰ for $\delta\ ^{13}C$ values, depending on all the nature of the food eaten and the discrimination of the enzymes involve in the biosynthesis pathways. Synthetic T is made from a plant species, mostly soy, with $\delta\ ^{13}C$ values of about -29‰, allowing differentiation of the human one.

The production of pregnenolone, an important intermediate of steroidal drug synthesis was reported from a simple carbon source (ethanol) by recombinant yeast *Saccharomyces cerevisiae* strains (1).

We studied the influence of carbon source on the resulting pregnenolone production from this engineered yeast. The carbon source came from ethanol with two different ^{13}C values (ethanol from sugar cane and from sugar beet). The results of $\delta\ ^{13}C$ determination were presented in the following Table:

Compounds	$^{13}C\ \delta$‰
Pregnenolone acetate from SIGMA Carbon source: plant sterols?	- 36.1
Pregnenolone acetate from Yeast Carbon source : Ethanol from sugar beet	- 19.0
Ethanol from sugar beet	- 26.5
Pregnenolone acetate from Yeast Carbon source : Ethanol from sugar cane	- 3.1
Ethanol from sugar cane	- 12.1

There was a strong discrimination of carbon incorporation into the biosynthetic recombinant yeast steroids. The $\delta\ ^{13}C$ was very different from the natural human steroids one.

More recently, hydrocortisone and cortisone were also produced by other recombinant yeast (2). Corticosteroids could easily be studied by GC-C-IRMS as androstan-tri-one derivatives (3), and, as anabolic steroids, the differentiation between synthetic and natural human ones was effective. The choice of 11-hydroxy-etiocholanolone as an ERCs for anabolic steroids GC-

Dr. Makoto Ueki
Head of the IOC/WADA Laboratory
Mitsubishi Kagaku Bio-Clinical Laboratories, Inc.
Japan

Dr. Michael Verkouteren
Research Chemist
National Institute of Standards and Technology
United States

Prof. Christina Wang
Program Director
Harbor-UCLA Medical Center
United States

Mr. Rich Wanninger
Communications & Public Affairs Director
USADA
United States

Ms. Andrea Wickerham
Legal Relations and Policy Director
The National Center for Drug Free Sport
United States

Dr. Prof. Mountian Wu
Head of the IOC/WADA Laboratory
China Doping Control Center
China

Mr. Richard Young
USADA Counsel
Holme, Roberts & Owen, LLP
United States

Dr. Mario Zorzoli
Official Doctor
Union Cycling Internationale (UCI)
Switzerland

going to be very difficult. What if the athlete from Kenya lives in America on Mc Donald's for one month, two, three months, or one year? Is that going to change what the values are? It is really very difficult.

FOURCROY
We put too much emphasis on diet and not enough emphasis on natural mutations. We certainly know there are differences in androgen metabolic pathways in several countries, and I think looking at it from what causes cancer. I think we put much too much emphasis on the diet; although, we know some diet does change. I would hate to think we do not look at the metabolic pathways that are really sped up in some countries.

KAZLAUSKAS
I agree we have to do that. We do not understand that, we do not know what those effects are, but the other thing of interest is that understanding the Kenyan's eat a lot of corn.

BOWERS
So, again there is nothing wrong with taking a cutoff that identifies too many people because presumably you have got a confirmation method that then sorts that out. So having said that what is your confirmation method after you do this?

KAZLAUSKAS
In terms of confirmation, we would actually go back to repeating this sort of experiment. We would also have a look at diols and use some of their values, I guess, from the literature. Because we don't have the extensive studies on the diols, so, we actually look at a number of different things before we could do that.

BOWERS
Based on what Dr. Fourcroy said, no matter what we pick for criteria there is somebody in that population that is somewhat disadvantaged. If you have a TE ratio of 3.5 and that is natural you take a little bit of T, and you are over the 6 limit. If you are 0.1 you take a whole bunch of T and never get close, so I am not too concerned about the thing that you showed with the exogenous and endogenous. In fact, in a way you have actually reversed the population that are advantaged and disadvantaged.

KAZLAUSKAS
There are still a lot of false negatives. I think we are increasing the false negative results rather than the false positive. Again everyone is concerned about false positives, but I think the result of all of this is showing that we

- There is a need for additional research funding in this area. Topics include the sensitivity and specificity of screening using A/E versus the Adiols.

Session 1:

Basics of GC-C-IRMS in Doping Control

Chair:

Don Catlin

REFERENCES

1. Preliminary results on the carbon isotope ratios of ketonic steroids in urines collected from different countries, A. Cawley, J. Rogerson, K. Rahman, G. Trout, and R. Kazlauskas, Recent Advances in Doping Analysis (11), Proceedings of the Manfred Donike Workshop 21st Cologne Workshop on Dope Analysis, 16th to 21st March 2003, ed W. Schänzer *et al*, Sport & Buch Strauss, Köln 2003, p183-193.
2. Expression of Differences in Isotopic Composition, U. Flenker and W. Schanzer, Advances in Doping Analysis (11), Proceedings of the Manfred Donike Workshop 21st Cologne Workshop on Dope Analysis, 16th to 21st March 2003, ed W. Schänzer *et al*, Sport & Buch Strauss, Köln 2003, p179-182.

Question & Answer Discussion

KAZLAUSKAS
We have just simply taken **A**, naturally occurring within the individual at –24, and you administer a **T** at –30. Then you have 80% of the **A** in that individual is exogenous, you do just a proportionation calculation with what the delta values should be. So that gives you the calculated delta value for difference and elimination. You can see there is a very marked difference (variation) in the actual values for each of those proportions depending on what the starting **A** value is in that individual.

HOUGHTON
I would like to question the need for a cut off value of -27. To me, the value in this technique is that each individual acts as their own control. I can see, the value in the population base is that you are showing steroid in the biosynthetic pathway, all having similar values. From that point on, the value is in the difference between the ERC and the analyte of interest. So why do you need to say the cutoff at -27?

KAZLAUSKAS
I agree with you. I forgot to say was we had this as our criteria, but we are now saying the absolute cutoff is probably not the way to go and, in fact, a difference protocol is far more useful. We need to now work out what the difference is the best value. Until we have all the data in for 1200 or so elite athletes and the statistician has done his job, we will not know what the difference should be. How do you calculate that? Do you have to take in to account the country the individual came from, but that in itself is really

going to be very difficult. What if the athlete from Kenya lives in America on Mc Donald's for one month, two, three months, or one year? Is that going to change what the values are? It is really very difficult.

FOURCROY
We put too much emphasis on diet and not enough emphasis on natural mutations. We certainly know there are differences in androgen metabolic pathways in several countries, and I think looking at it from what causes cancer. I think we put much too much emphasis on the diet; although, we know some diet does change. I would hate to think we do not look at the metabolic pathways that are really sped up in some countries.

KAZLAUSKAS
I agree we have to do that. We do not understand that, we do not know what those effects are, but the other thing of interest is that understanding the Kenyan's eat a lot of corn.

BOWERS
So, again there is nothing wrong with taking a cutoff that identifies too many people because presumably you have got a confirmation method that then sorts that out. So having said that what is your confirmation method after you do this?

KAZLAUSKAS
In terms of confirmation, we would actually go back to repeating this sort of experiment. We would also have a look at diols and use some of their values, I guess, from the literature. Because we don't have the extensive studies on the diols, so, we actually look at a number of different things before we could do that.

BOWERS
Based on what Dr. Fourcroy said, no matter what we pick for criteria there is somebody in that population that is somewhat disadvantaged. If you have a TE ratio of 3.5 and that is natural you take a little bit of T, and you are over the 6 limit. If you are 0.1 you take a whole bunch of T and never get close, so I am not too concerned about the thing that you showed with the exogenous and endogenous. In fact, in a way you have actually reversed the population that are advantaged and disadvantaged.

KAZLAUSKAS
There are still a lot of false negatives. I think we are increasing the false negative results rather than the false positive. Again everyone is concerned about false positives, but I think the result of all of this is showing that we

- There is a need for additional research funding in this area. Topics include the sensitivity and specificity of screening using A/E versus the Adiols.

- The $\delta^{13}C$ values obtained for **A**, **E** and 11-keto were found by ANOVA analysis to be significantly different ($p<0.0001$) for Kenya, China and Australia/New Zealand.
- The distributions are close to normal with some differences in standard deviations for each country.
- There is a consistent difference of approximately 2‰ between the average of **A** and **E** and the ERC.
- The most negative $\delta^{13}C$ value found for **A** is –25.1‰ and for **E** is –25.8‰. Thus the absolute cutoff of –27.0‰ is supported.
- The use of ratio of $\delta^{13}C$ values to ERC is not supported as the ratio is dependent on the size of the values measured (1,2)
- Athletes with higher $\delta^{13}C$ values for the **A**, **E** and ERC are advantaged relative to those with lower values as more exogenous material is needed for them to fail all criteria (difference to ERC and absolute value). However they are more sensitive to the difference criteria alone.
- The absolute cutoff of –27.0‰ may be reasonable for Australian and New Zealand populations but may not be appropriate for populations with higher d13C values such as the Kenyan population. A possible general rule is to define doping as a difference of 6‰ or greater or a difference of 4‰ or greater providing the $\delta^{13}C$ values of **A** and **E** are more negative than -27.0‰.
- These values only apply if 11-ketoetiocholanolone is used as the ERC. Using pregnanediol or other ERC markers gives different values for the difference cutoff and population studies using those markers need to be performed.

Table summarizing the $\delta^{13}C$ data for the various countries.

Country	Number	Mean Etio ‰	Mean Andro ‰	Mean 11-keto ‰
Australia	59	-23.0 SD 0.97 Min -25.3	-22.5 SD 1.16 Min -24.6	-21.1 SD 1.29 Min -24.1
New Zealand	108	-23.3 SD 1.07 Min -25.8	-23.0 SD 1.16 Min -25.1	-20.9 SD 1.21 Min -23.8
China	157	-22.5 SD 1.29 Min -25.0	-21.6 SD 1.53 Min -25.0	-20.1 SD 1.59 Min -24.0
Kenya	127	-20.5 SD 1.53 Min -24.0	-20.3 SD 1.38 Min -23.0	-18.6 SD 1.81 Min -24.0

Human Anabolic/Androgenic Steroid Synthesis and Metabolism

Richard Auchus, M.D.
University of Texas Southwestern Medical Center

Steroid Biosynthesis:

The Five Components of Steroidogenesis:

1. The conversion of cholesterol to pregnenolone. The mobilization of cholesterol into the steroidogenic pathways is a complex event that not only serves as a key locus of acute regulation but also conventionally defines a tissue as "steroidogenic"—the adrenal cortex, ovarian theca cells, and trophoblast cells of the placenta.
2. The transformation of pregnenolone to active hormones, intermediates and exported steroid derivatives. The repertoire of enzymes and cofactor proteins present in a given steroidogenic cell generates the characteristic steroid profile of that cell type, and the coordinate regulation of their expression promotes the completion of all steps of a given pathway. Thus, these enzymes determine qualitatively *what* steroids are made, but since these steps are not kinetically limiting, it is Step 1 that quantitatively regulates *how much* steroid is made at a given moment.
3. Peripheral metabolism of hormones and precursors. Although not "steroidogenic" as defined above, some organs like the liver and skin possess tremendous capacity to transform a variety of steroids. For example, ~80% of circulating **T** in normal menstrual cycle women derives from the conversion of adrenal DHEA.
4. Target tissue metabolism. Steroids can be activated in target tissues, such as the conversion of **T** by steroid 5α-reductase, type 2, to dihydroT in the prostate. In contrast, active androgens and estrogens are inactivated in the uterus and other peripheral tissues by 17 β hydroxysteroid dehydrogenase (17βHSD), type 2.
5. Catabolism and unproductive metabolism. A panopoly of steroids can be isolated from human plasma and tissues, many of which have negligible biological activity. Most inactive by-products derive from hepatic transformations, such as 6α/6β-hydroxylation of C_{19} steroids, which promote renal excretion of these steroids.

Types of Enzymes

Cytochromes P450. P450 enzymes activate molecular oxygen using their heme center and electrons from Nicotinamide-Adenine Dinucleotide Phosphate (NADPH) via "redox partners" (flavo- or

metallo-proteins). P450 reactions on steroids are limited to oxygen insertion (hydroxlyation) reactions and, in a few notable cases, oxidative carbon-carbon bond cleavage reactions. P450 reactions irreversibly oxidize and/or remove carbon atoms from the steroid nucleus, and thus an organized sequence of these reactions forms the traditional pathways of steroid synthesis.

Hydroxysteroid Dehydrogenases (HSDs) and Reductases. All HSDs and related enzymes use nicotinamide cofactors either to reduce or to oxidize the steroid by two electrons with two hydrogen atoms. Most examples involve the conversion of a secondary alcohol to a ketone or vice-versa, and in the case of the 3β-hydroxysteroid dehydrogenase/$\Delta^{5/4}$-isomerases (3βHSDs), the dehydrogenation is accompanied by the isomerization of the adjacent carbon-carbon double bond from the Δ^5 (between carbons 5 and 6) to the Δ^4 positions. A few members of this group, such as the steroid 5α- and 5β-reductases, reduce olefinic carbon-carbon double bonds to the saturated state rather than acting on oxygenated carbon centers.

The HSDs can be broadly dichotomized according to either structural or functional classifications. *Structurally*, HSDs are members of either the short-chain dehydrogenase reductase (SDR) or aldo-keto reductase (AKR) families. Human 17βHSDs types 1-3 are SDR enzymes, and they are efficient, selective, membrane-bound enzymes. The reductive 3α-HSDs and 17β-HSD5 are AKR enzymes, and these are relatively slow, promiscuous, and soluble proteins. *Functionally*, HSDs act either as true dehydrogenases, using Nicotinamide-Adenine Dinucleotide $(NAD)^+$ as cofactor to convert hydroxysteroids to ketosteroids, or as ketosteroid reductases, utilizing predominantly NADPH to reduce ketosteroids. The preferred direction of each enzyme is determined by cofactor binding, because cofactor concentrations exceed steroid concentrations by many orders of magnitude. The reactions catalyzed by key isoforms of these enzymes are shown in the Figures 1 & 2.

Peripheral Metabolism

Concepts

It is important to distinguish irreversible from reversible enzymatic transformations. Reversible processes dynamically influence steroid potency in the plasma and in cells. The end-products of steroid metabolism excreted in the urine are determined by the irreversible transformations that tend to drive steroids "downhill" to inactive

Some preliminary results on the carbon isotope ratios of ketonic steroids in urines collected from different countries

Adam Cawley, Jill Rogerson, Kishwar Rahman, Graham Trout, and Ray Kazlauskas
Australian Sports Drug Testing Laboratory

This talk was given to provide some information on the variation of the Carbon Isotope Ratio for different countries. We measure the $\delta^{13}C$ values of **A** and **E** in urine as indicators of administration of exogenous source of an anabolic steroid which may also occur naturally. This procedure works best for the "prohormones" AED, DHEA and the androstenediols. It also is a good indicator of **T** use but mainly for recent application of the drug.

In early 2000 as part of the EPO2000 project blood and urine samples were collected from some 1200 elite athletes around the world. The ethics approval for this project included analysis of the urine samples for steroids and in particular CIR measurements. The use of these samples allows us to get a good insight into the variability of the CIR values for a number of countries where diet may be the major contributor to any variability (1).

We use an endogenous internal marker (ERC) which is a compound that occurs through a different metabolic pathway and which is not affected by administration of **T** or its immediate precursors. The internal marker (11-ketoetiocholanolone) thereby represents the natural value of the CIR for that individual and marked difference from that marker in a direction towards the CIR of synthetic steroids indicates application of a drug. This internal marker is useful as it often occurs in easily measurable amounts. Typical internal marker concentrations are from 30% to 50% of the **A** concentration using our sample preparation procedure. The data we have accumulated over the past years allows a set of criteria to be established in order to allow a sample to be reported as being outside the normal range:

- The difference between the average of $\delta^{13}C$ for **A** and **E** and the $\delta^{13}C$ for the 11-ketoetiocholanolone (11-keto) must be greater than 4‰.
- The $\delta^{13}C$ for **A** and **E** must be more negative than –27.0‰ (3).

The results obtained for the samples collected from Australia, New Zealand, China and Kenya give insight into the applicability of the above criteria and are presented here. A summary of the findings is set out in the table below.

The data can be summarized as follows:

- The $\delta^{13}C$ values obtained for **A**, **E** and 11-keto were found by ANOVA analysis to be significantly different ($p<0.0001$) for Kenya, China and Australia/New Zealand.
- The distributions are close to normal with some differences in standard deviations for each country.
- There is a consistent difference of approximately 2‰ between the average of **A** and **E** and the ERC.
- The most negative $\delta^{13}C$ value found for **A** is –25.1‰ and for **E** is –25.8‰. Thus the absolute cutoff of –27.0‰ is supported.
- The use of ratio of $\delta^{13}C$ values to ERC is not supported as the ratio is dependent on the size of the values measured (1,2)
- Athletes with higher $\delta^{13}C$ values for the **A**, **E** and ERC are advantaged relative to those with lower values as more exogenous material is needed for them to fail all criteria (difference to ERC and absolute value). However they are more sensitive to the difference criteria alone.
- The absolute cutoff of –27.0‰ may be reasonable for Australian and New Zealand populations but may not be appropriate for populations with higher d13C values such as the Kenyan population. A possible general rule is to define doping as a difference of 6‰ or greater or a difference of 4‰ or greater providing the $\delta^{13}C$ values of **A** and **E** are more negative than -27.0‰.
- These values only apply if 11-ketoetiocholanolone is used as the ERC. Using pregnanediol or other ERC markers gives different values for the difference cutoff and population studies using those markers need to be performed.

Table summarizing the $\delta^{13}C$ data for the various countries.

Country	Number	Mean Etio ‰	Mean Andro ‰	Mean 11-keto ‰
Australia	59	-23.0 SD 0.97 Min -25.3	-22.5 SD 1.16 Min -24.6	-21.1 SD 1.29 Min -24.1
New Zealand	108	-23.3 SD 1.07 Min -25.8	-23.0 SD 1.16 Min -25.1	-20.9 SD 1.21 Min -23.8
China	157	-22.5 SD 1.29 Min -25.0	-21.6 SD 1.53 Min -25.0	-20.1 SD 1.59 Min -24.0
Kenya	127	-20.5 SD 1.53 Min -24.0	-20.3 SD 1.38 Min -23.0	-18.6 SD 1.81 Min -24.0

Human Anabolic/Androgenic Steroid Synthesis and Metabolism

Richard Auchus, M.D.
University of Texas Southwestern Medical Center

Steroid Biosynthesis:

The Five Components of Steroidogenesis:

1. The conversion of cholesterol to pregnenolone. The mobilization of cholesterol into the steroidogenic pathways is a complex event that not only serves as a key locus of acute regulation but also conventionally defines a tissue as "steroidogenic"—the adrenal cortex, ovarian theca cells, and trophoblast cells of the placenta.
2. The transformation of pregnenolone to active hormones, intermediates and exported steroid derivatives. The repertoire of enzymes and cofactor proteins present in a given steroidogenic cell generates the characteristic steroid profile of that cell type, and the coordinate regulation of their expression promotes the completion of all steps of a given pathway. Thus, these enzymes determine qualitatively *what* steroids are made, but since these steps are not kinetically limiting, it is Step 1 that quantitatively regulates *how much* steroid is made at a given moment.
3. Peripheral metabolism of hormones and precursors. Although not "steroidogenic" as defined above, some organs like the liver and skin possess tremendous capacity to transform a variety of steroids. For example, ~80% of circulating **T** in normal menstrual cycle women derives from the conversion of adrenal DHEA.
4. Target tissue metabolism. Steroids can be activated in target tissues, such as the conversion of **T** by steroid 5α-reductase, type 2, to dihydro**T** in the prostate. In contrast, active androgens and estrogens are inactivated in the uterus and other peripheral tissues by 17 β hydroxysteroid dehydrogenase (17βHSD), type 2.
5. Catabolism and unproductive metabolism. A panopoly of steroids can be isolated from human plasma and tissues, many of which have negligible biological activity. Most inactive by-products derive from hepatic transformations, such as 6α/6β-hydroxylation of C_{19} steroids, which promote renal excretion of these steroids.

Types of Enzymes

Cytochromes P450. P450 enzymes activate molecular oxygen using their heme center and electrons from Nicotinamide-Adenine Dinucleotide Phosphate (NADPH) via "redox partners" (flavo- or

Session 4:

Harmonization of Inter-laboratory Results

Chair:

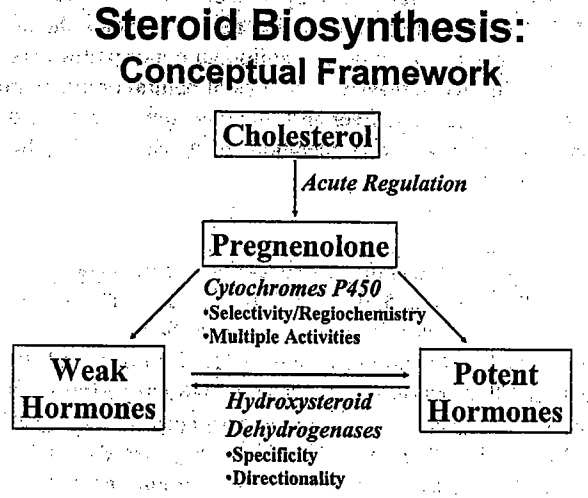
Richard Hilderbrand

compounds that are first oxidized (hydroxylated and/or cleaved by P450s) and then reduced (5α- or 5β-reduced 3, 17-hydroxysteroids). The 17βHSD and 3αHSD reactions are reversible, or at least have pairs of enzymes that preferentially run in the oxidative and reductive directions; the additional isomerization step renders the 3βHSD enzymes irreversible. The 5α- and 5β-reductases are irreversible, as are all P450-mediated oxygenations. Figures 1 & 2 detail the major pathways in normal physiology, but additional minor pathways can become significant when massive amounts of natural or unnatural steroids are consumed. The liver and skin have the greatest capacity to metabolize androgens, but local metabolism can also regulate hormone potency.

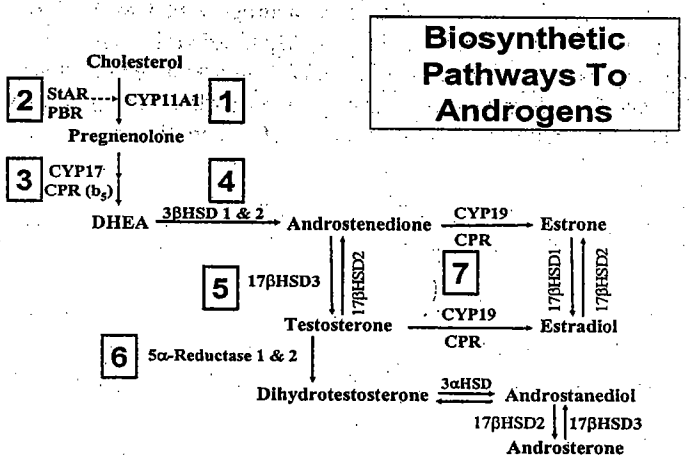
Enzymes

The key enzymes that mediate peripheral C_{19} steroid metabolism are 3βHSD1, 17βHSDs 2 and 5, the 3αHSDs, the 5α- and 5β-reductases, and CYP3A4. All Δ^5 steroids are converted to their Δ^4 congeners by 3βHSD1 (i.e. DHEA to androstenedione [AED]) in liver and skin. The oxidative 17βHSD2 converts T to AED in multiple peripheral tissues, and 17βHSD5 converts AED to T in the periphery, whereas 17βHSD3 converts AED to T only in the testis. These enzymes also metabolize AED and 5α-dihydrotestosterone (DHT) in a similar manner. Analogously, the oxidative and reductive 3αHSDs interconvert DHT and Adiol. The 5α- and 5β-reductases convert T to DHT and etiocholan-17β-ol-3-one, respectively. Finally, hepatic P450s, mainly CYP3A4, 6α- and 6β-hydroxylate many androgens such as A. For these reasons, many precursors (DHEA, AED, and variants on these structures) can be metabolized to T and DHT and excreted in the urine as 5α- and 5β-Adiols or as A and E. Also, 6α-hydroxy metabolites are common, especially when large amounts of C_{19} steroids are ingested orally.

Figure 1: (Top) The conceptual framework of how different enzymes and tissues participate in steroid biosynthesis and metabolism. Many aspects of the 5 components of steroidogenesis can all be incorporated into this scheme.



(Bottom) The principal enzymes involved in the biosynthesis of the active androgens T and DHT. Some subsequent products, the estrogens and A + Adiol, are included for completeness.



The following points were emphasized:

- Well-separated peaks with good symmetry are important to analysis.
  - Contributions of system components to peak broadening and tailing
  - Potential for use of programmable temperature injection (PTI) to improve peak shape and increase amount of compound applied to the column
- There are differences between results based on instrumental approach to peak integration and calibration.
  - Importance of integration algorithm (e.g., summation)
  - Potential for application of peak fitting to improve results
  - Data reduction with a single algorithm independent of the instrument
- Characterization of peak envelope – width, height, resolution, shoulders, asymmetry – and development of criteria for acceptable peak characteristics are important.
- Differences between elemental analysis and GC-C-IRMS values for the same compound create concerns about purity.

Session 4:

Harmonization of Inter-laboratory Results

Chair:

Richard Hilderbrand

compounds that are first oxidized (hydroxylated and/or cleaved by P450s) and then reduced (5α- or 5β-reduced 3, 17-hydroxysteroids). The 17βHSD and 3αHSD reactions are reversible, or at least have pairs of enzymes that preferentially run in the oxidative and reductive directions; the additional isomerization step renders the 3βHSD enzymes irreversible. The 5α- and 5β-reductases are irreversible, as are all P450-mediated oxygenations. Figures 1 & 2 detail the major pathways in normal physiology, but additional minor pathways can become significant when massive amounts of natural or unnatural steroids are consumed. The liver and skin have the greatest capacity to metabolize androgens, but local metabolism can also regulate hormone potency.

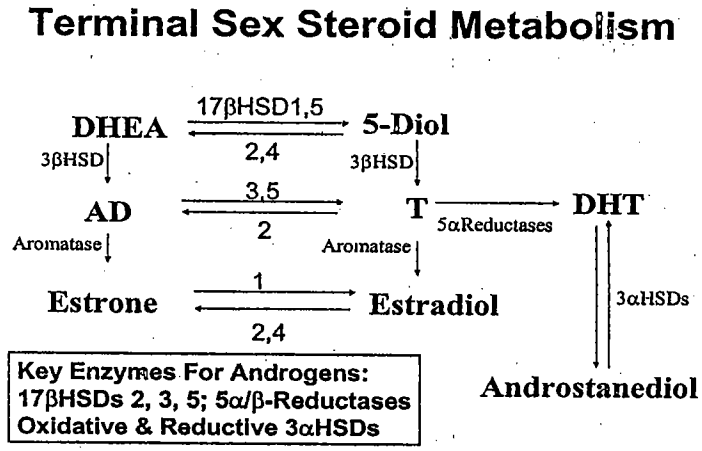
Enzymes

The key enzymes that mediate peripheral C_{19} steroid metabolism are 3βHSD1, 17βHSDs 2 and 5, the 3αHSDs, the 5α- and 5β-reductases, and CYP3A4. All Δ^5 steroids are converted to their Δ^4 congeners by 3βHSD1 (i.e. DHEA to androstenedione [AED]) in liver and skin. The oxidative 17βHSD2 converts **T** to AED in multiple peripheral tissues, and 17βHSD5 converts AED to T in the periphery, whereas 17βHSD3 converts AED to **T** only in the testis. These enzymes also metabolize AED and 5α-dihydrotestosterone (DHT) in a similar manner. Analogously, the oxidative and reductive 3αHSDs interconvert DHT and Adiol. The 5α- and 5β-reductases convert T to DHT and etiocholan-17β-ol-3-one, respectively. Finally, hepatic P450s, mainly CYP3A4, 6α- and 6β-hydroxylate many androgens such as A. For these reasons, many precursors (DHEA, AED, and variants on these structures) can be metabolized to T and DHT and excreted in the urine as 5α- and 5β-Adiols or as **A** and **E**. Also, 6α-hydroxy metabolites are common, especially when large amounts of C_{19} steroids are ingested orally.

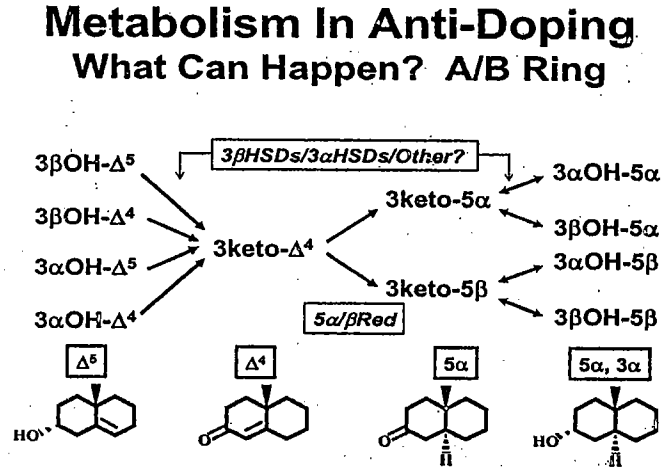
Summary of Discussions:

Uncertainty in GC-C-IRMS Measurement as Applied to Doping Control

Figure 2: (Top) The HSD enzymes involved in the terminal stages of sex steroid (androgen and estrogen) biosynthesis and catabolism.



(Bottom) A generic scheme for the metabolic fates of C_{19} steroids with various A/B ring systems. The Δ^5 steroids can be metabolized to Δ^4 steroids by 3βHSD1.. The Δ^4 steroids are substrates for both the 5α- and 5β-reductases. Once the Δ^4 double bond has been saturated, these 3-ketosteroids can be further reduced, primarily by reductive 3αHSDs.



Question & Answer Discussion

COWAN
Thank you. It was a very interesting presentation. I would be very interested to hear your thoughts on the origin of epitestosterone (epi-T).

AUCHUS
I believe that it is an enzyme that we already know; we just don't know that it does it. It is probably one of the aldo-keto reductases; it may not be one of the aldo-keto reductase that we traditionally associate with steroid metabolism. There are now 240 aldo-keto reductase genes. This is one of the projects that Dr. Wilson and I have talked about doing. I would bet that it comes from 17 keto-steroid, there must be 3α-HSDs , and probably an aldo-keto reductase. Some of these aldo-keto reductases will be both 3α-HSDs and 20β –HSDs, so in other words, if you flip the steroid upside down it will work in the other position as well. So, it may be a 3β aldo-keto reductase that is doing 17α.

HALE
It is very good to hear interesting suppositions in a couple places there. Let's go back to the female. What happens if you overload in the female end organ system with the estrogen compound; are you saying that you can drive that back to T and that it would be a normal occurring T?

AUCHUS
No, no, no. The P450's are irreversible. With CYP19, which is an aromatase, it cleaves the C_{19} off. Once it is off it does not go back on. There are some compounds that have been said to have some androgenic activity that have an aromatic A ring, but the evidence is very weak. So you can covert androgens that have a C_{19} to estrogens, but you can't go back in the other direction. Some textbooks still talk about estrogen to androgen conversion, but it just doesn't happen.

Another question that is interesting in that regard. How do women make T? Because they do, and they do not have the type 3 enzyme. The type 3 enzyme is only found in the testes. Everybody thinks it is the type 5 enzyme, which I have already told you is a lousy enzyme. If you put the type 2 enzyme in the presence of AED, it will go back 2% to T. We actually think it is the type 2 enzyme that is supposed to inactivate it that is actually equilibrating a little percentage of it. If it is true, that

performed gas chromatographic separation ideally resulting in base line separation, small peak widths and high peak symmetry. Furthermore the transfer of the analytes from the GC to the ion source via the open split and the combustion furnaces and the mass spectrometers themselves should be optimized where possible.

Choice of the method validation procedure

Validation of a method to International standards through a collaborative trial requires a method protocol to be followed very closely. The urinary studies are intended to provide such a method for validation by the primary partners in the ISOSTER project. However, extension of the validation to other laboratories is necessary according to the International standards Association of Analytical Communities (AOAC), International Union of Pure & Applied Chemistry (IUPAC).

As a consequence of this the attendees of the symposium are invited to encourage their laboratories to participate in the ISOSTER method validation study.

Question & Answer Discussion

UEKI
How does Dr. Preiss-Weigert express peak shape in writing? Do we have to calculate HETP in each run?

PREISS-WEIGERT
I cannot tell you. I have not designed the study, and I think they have not really defined the values for peak shape, which data they want to get back.

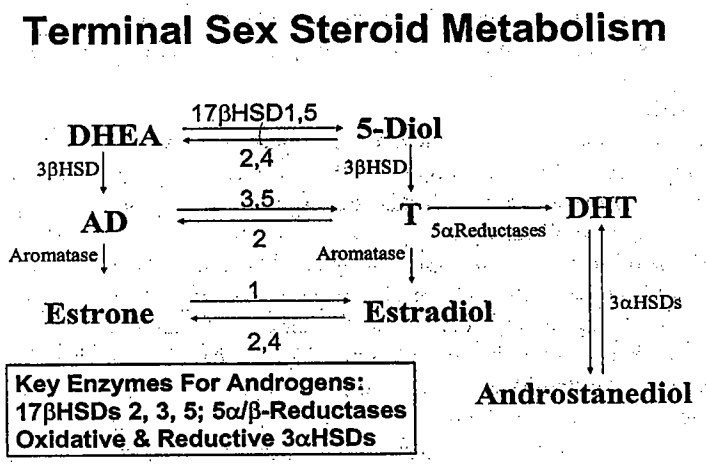
UEKI
The peak shape is very important, but I have no idea of how to express peak shape in writing.

PREISS-WEIGERT
The peak width of course and the symmetry of the peak, but they are the fundamentals.

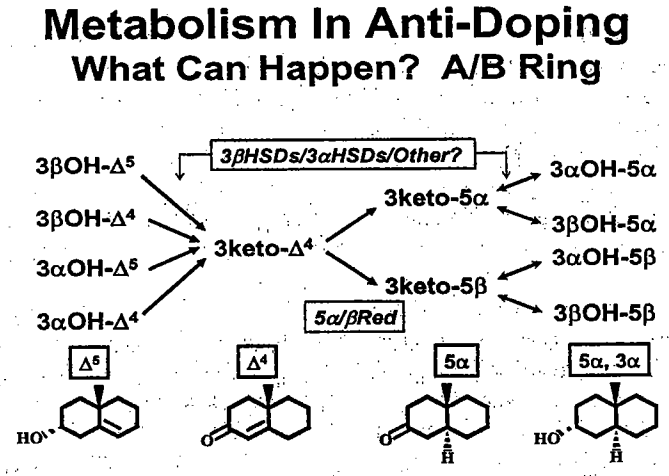
Summary of Discussions:

Uncertainty in GC-C-IRMS Measurement as Applied to Doping Control

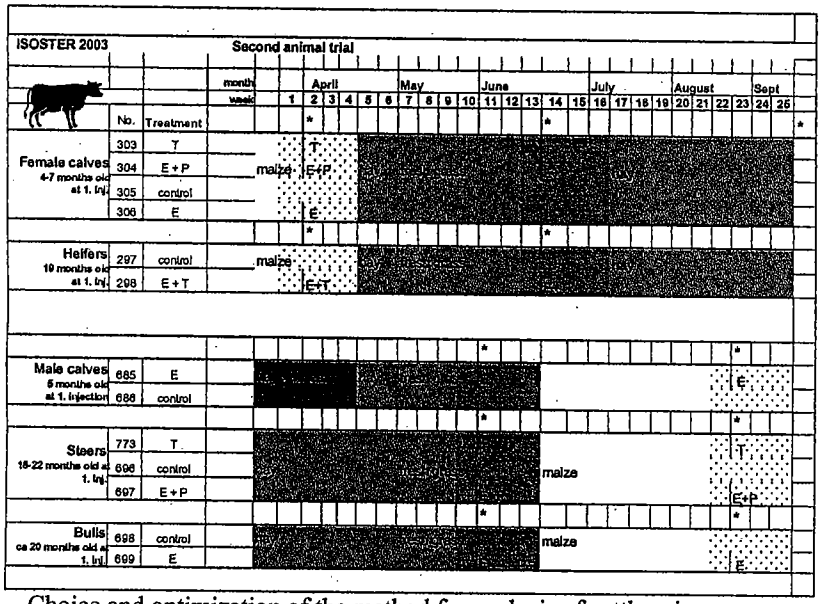
Figure 2: (Top) The HSD enzymes involved in the terminal stages of sex steroid (androgen and estrogen) biosynthesis and catabolism.



(Bottom) A generic scheme for the metabolic fates of C_{19} steroids with various A/B ring systems. The Δ^5 steroids can be metabolized to Δ^4 steroids by 3βHSD1. The Δ^4 steroids are substrates for both the 5α- and 5β-reductases. Once the Δ^4 double bond has been saturated, these 3-ketosteroids can be further reduced, primarily by reductive 3αHSDs.



Animal trial aiming at the comparison of the influences of different feeding regimen



Choice and optimization of the method for analysis of cattle urine

The project partners agreed to select the method developed by LABERCA, Nantes, Fr, as starting point for the method validation procedure. Optimization at several levels of the sample preparation is currently underway and involves the conditions of the deconjugation step, the SPE procedures, the HPLC purification system as well as the derivatization procedure.

The most interesting aspect will probably be the comparison of different types of derivatives. Matters for consideration will be possible fractionation effects during the derivatization reaction, their chromatographic properties, their signal intensities in the IRMS, the precision of the measured δ-values and finally their behaviour in a large volume injection system (PTV).

The quality of the measurements also depends upon the equipment and the skill in its application. Therefore the partners are supported by the manufacturers of the IRMS devices. Their input focuses on increasing the sensitivity of the measurement. Attention has to be paid to a thoroughly

we can modulate that equilibrium to constant by modulating cellular cofactors, there can be conditions where more of it is generated by this very good enzyme that is found everywhere. '

AGUILERA
How much evidence do you have of the transformation from Adiol to DHT?

AUCHUS
Of the oxidative 3α-HSDs, which can do that conversion, Adiol back to DHT, there are three in the human. Dr. David Russell has cloned one of those enzymes from prostate and genital skin. They are in the Retinol DeHydrogenase (RoDH) family. Because they are also retinal dehydrogenases; there are three of them. Dr. Stefan Anderson and I are starting to work on those as well. They are pretty efficient. They are certainly located in genital skin. Which is again, why we think this back door path to Adiol may be important during fetal development. How much capacity they have, we are not so sure of.

In the liver, the reductive 3α-HSDs dominate, which would convert DHT to Adiol. We can also make those enzymes go backwards by NADPH completion. We don't really have a good sense now of what the capacity of those enzymes to metabolize systemic loads of these steroids are. Certainly local amounts, there are plenty of capacity in the genital tissue. In terms of generating systemically large circulating pools; we don't really know the answer to that.

CATLIN
One of the things that sportsmen do is take big doses. They also take epi-T. Can you speculate what somebody would do if they took a huge dose of epi-T, either clinically or biochemically?

AUCHUS
If there were a 3α-HSD that can make it, then because, these enzymes really are reversible, you ought to go back to AED. Then that should proportionate between epi-T and T. I would think that the absolute amount of the T would go up. But how much relative to the Epi-T? Can you really use the ratio? That is why I am saying, we have to get a sense of abundance in addition to ratios here.

Fundamentals of compound identification and measurement using isotopes and mass spectrometry

Dwight Matthews, Ph.D.
University of Vermont

Detection of banned substances in vivo from blood, urine, or other physiological sampling falls into two areas: (i) detection of administered compounds that are not produced in the body (exogenous compounds administered exogenously, such as drugs) and (ii) detection of compounds that are produced in the body (endogenous compounds) that are administered exogenously. Detection of non-endogenous drugs is much simpler than detection of addition of endogenous compounds that are administered to produce a "drug" effect. Mass spectrometry has proven to be an extremely reliable, sensitive, and precise technique for measuring exogenous drugs in physiological samples. The primary techniques used are gas chromatography-mass spectrometry (GCMS) and, more recently, liquid chromatography-mass spectrometry (LCMS). These techniques form the basis for most measurement of drugs and drug metabolites in physiological samples.

Measurement of exogenously administered *endogenous* compounds is a much more difficult task. Because levels of most metabolites vary among individuals and metabolic state (e.g. fasting v. fed or time of day), simple measurement of an abnormally elevated concentration of a metabolite in the body or urine does not necessarily represent exogenous administration. Compounds with long half lives or which produce metabolites with long half lives are easier to define and detect on the basis of elevated concentrations. Compounds with rapid turnover in the body are difficult to detect.

An alternative approach is detecting exogenously administered compounds that exist normally as endogenous compounds using physicochemical differences between the exogenously administered and endogenously produced material. The physicochemical differences that are available are differences in natural abundance of isotopes in the two sets of compound that arise on the basis of the different synthetic pathways. Most exogenously administered compounds are made in laboratories; very few compounds are administered after isolation from an animal source. As such, the synthetic routes and the starting materials of synthesis are different from those produced endogenously.

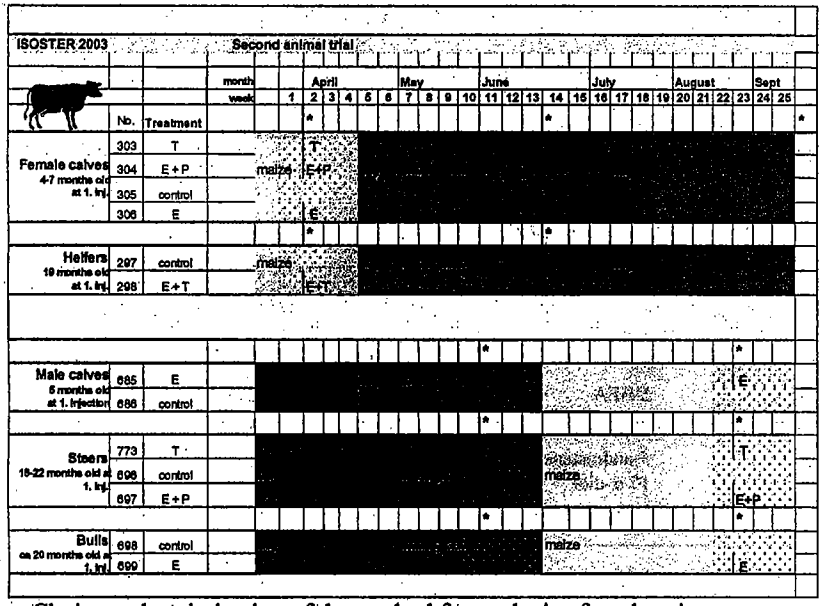
The presentation at this conference is focused on aspects of the ISOSTER project dealing with the analysis of urine samples. The presentation addresses the animal trials, the choice and the optimization of the analytical method and finally discusses the schedule of the method validation procedure.

Initially, the analytes for the measurements in urine had to be selected. The selection of the precursors and the target analytes depended on the concentration, which has to be in a sufficient amount to be measurable by GC-C-IRMS.

Selection of analytes for urine

Treatment	PERC	Target Analyte
EstrAdiol	DHEA	17 α-estrAdiol
	5-androstene-3 β, 17 α-diol	Estrone
T	DHEA	5 α-androstane-3 β,17 α-diol
	5-androstene-3 β, 17 α-diol	Etiocholanolone
		Epi-T

Animal trial aiming at the comparison of the influences of different feeding regimen



Choice and optimization of the method for analysis of cattle urine

The project partners agreed to select the method developed by LABERCA, Nantes, Fr, as starting point for the method validation procedure. Optimization at several levels of the sample preparation is currently underway and involves the conditions of the deconjugation step, the SPE procedures, the HPLC purification system as well as the derivatization procedure.

The most interesting aspect will probably be the comparison of different types of derivatives. Matters for consideration will be possible fractionation effects during the derivatization reaction, their chromatographic properties, their signal intensities in the IRMS, the precision of the measured δ-values and finally their behaviour in a large volume injection system (PTV).

The quality of the measurements also depends upon the equipment and the skill in its application. Therefore the partners are supported by the manufacturers of the IRMS devices. Their input focuses on increasing the sensitivity of the measurement. Attention has to be paid to a thoroughly

we can modulate that equilibrium to constant by modulating cellular cofactors, there can be conditions where more of it is generated by this very good enzyme that is found everywhere.

AGUILERA
How much evidence do you have of the transformation from Adiol to DHT?

AUCHUS
Of the oxidative 3α-HSDs, which can do that conversion, Adiol back to DHT, there are three in the human. Dr. David Russell has cloned one of those enzymes from prostate and genital skin. They are in the Retinol DeHydrogenase (RoDH) family. Because they are also retinal dehydrogenases; there are three of them. Dr. Stefan Anderson and I are starting to work on those as well. They are pretty efficient. They are certainly located in genital skin. Which is again, why we think this back door path to Adiol may be important during fetal development. How much capacity they have, we are not so sure of.

In the liver, the reductive 3α-HSDs dominate, which would convert DHT to Adiol. We can also make those enzymes go backwards by NADPH completion. We don't really have a good sense now of what the capacity of those enzymes to metabolize systemic loads of these steroids are. Certainly local amounts, there are plenty of capacity in the genital tissue. In terms of generating systemically large circulating pools; we don't really know the answer to that.

CATLIN
One of the things that sportsmen do is take big doses. They also take epi-T. Can you speculate what somebody would do if they took a huge dose of epi-T, either clinically or biochemically?

AUCHUS
If there were a 3α-HSD that can make it, then because, these enzymes really are reversible, you ought to go back to AED. Then that should proportionate between epi-T and T. I would think that the absolute amount of the T would go up. But how much relative to the Epi-T? Can you really use the ratio? That is why I am saying, we have to get a sense of abundance in addition to ratios here.

ISOSTER – Determination of the Origin of Hormones in Cattle

Angelika Preiss-Weigert[+1], Hildburg Fry[+1], Stefanie Banneke[+1], Bruno Le Bizec[+2], Wilhelm Schänzer[+3], Ed Houghton[+4] and Matthew Sharman[+5]

[+1] BfR, Berlin, D
[+2] LABERCA, ENV, Nantes, F
[+3] DSHS, Cologne, D
[+4] HFL, Fordham, UK
[+5] CSL, York, UK

The ISOSTER project is funded by the EC within the Growth programme as a part of the 5th framework programme. Four main partners (BfR, LABERCA, DSHS and CSL) are assisted by the Queens University in Belfast (QUB), UK, and by the TNO in Zeist, NL. Furthermore an added advantage is that the project involves three industrial partners: Thermo Finnigan, Bremen, D; GV Instruments, Manchester, UK; and DIONEX, Idstein, D.

The long term aim of the ISOSTER project is the creation of a tool for food control laboratories to allow them to control the administration of natural steroid hormones as growth promoting agents in food production.

The usual sampling locations of food control determine the choice of the matrix for analysis: as a consequence of this, methods applying GC-C-IRMS has to be developed for urine, feces, muscle, kidney, and fat samples. Additionally a major objective of this project is the validation of the GC-C-IRMS method for urine samples.

A further objective is the preparation of authentic samples derived from animals with a known history and kept under controlled conditions. In several animal trials, performed at the animal farm of the BfR in Berlin, samples from different categories of cattle (females and males, young animals and adults) have been collected. Thoroughly designed conceptual plans for the animal trials, taking into consideration the influences of feeding, should allow the variation of isotopic data of the steroids with and without administration of steroid hormones to be determined.

The aim of this project is to assess the effectiveness of the developed GC-C-IRMS methods for the differentiation between the endogenous and exogenous origin of steroids in cattle.

Isotope geochemists and chemists studying organic reaction mechanisms have studied isotope effects of chemical reactions extensively. The isotope effects are generally proportional to the square root difference between the two isotopes being studied. For example, the isotope effect of deuterium from protium is in the range of $^{2}H/^{1}H = [2/1]^{½} - 1 = 41\%$ or more, while that of $^{13}C/^{12}C = [13/12]^{½} - 1 = 4\%$ or less. The isotope effects seen in nature approximate these differences in mass alone. Although significantly greater natural differences will be found for hydrogen, due to its larger isotope effects, use of isotope effects to detect exogenously given metabolites has centered on measuring isotope differences of carbon because methods of measurement of physiological samples have been developed more fully. Isotope ratio mass spectrometry (IRMS) was developed in the 1930's for measurement of light element isotopes and saw immediate application to measurement of isotopes of carbon, nitrogen, oxygen, and hydrogen.

The hydrogen isotopes have always been the most problematic to measure, while carbon isotopes of organic compounds have been the simplest because the material to be measured only has to be purified and oxidized to CO_2 for measurement by IRMS. The only drawback is that purification and isolation of a metabolite in small amounts from a physiological sample is not a simple task. In 1978 Matthews and Hayes, demonstrated a new technique that eventually came to be known as gas chromatography-combustion-IRMS (GC-C-IRMS). The technique put a GC as the front end of the mass spectrometer and inserted an oxidation oven between the GC and an IRMS to oxidize all material eluting from the GC to CO_2. This technique took advantage of the reduction of noise by measuring all ^{13}C as CO_2 and measuring all samples against a reference CO_2 gas of known isotopic content. Since the initial 1978 report, a number of developments (e.g. introduction of fused silica GC columns) made commercial instruments feasible, and many laboratories around the world now have GC-C-IRMS instruments as their primary approach to IRMS measurement.

The GC-C-IRMS measurement for carbon measures the $^{13}CO_2/^{12}CO_2$ ratio for eluting compounds from the GC against a standard reference CO_2 gas of known ^{13}C content. Thus, the common values reported from the GC-C-IRMS are ^{13}C values in delta (δ) units in per mil (‰):

$$\delta = [R/R_{std} - 1] \cdot 1000$$

where R is the $^{13}C/^{12}C$ ratio of the measured sample peak and R_{std} is the $^{13}C/^{12}C$ ratio of the accepted Vienna PeeDee Belemnite (VPDB) limestone standard (note: the actual δ that the instrument produces is referenced to a laboratory standard gas used for that particular instrument, then is referenced to the VPDB primary standard). Most practitioners express their results directly in δ units – even if these measurements do not have a direct connection to ^{13}C enrichment and are referenced to a non-physiological standard (limestone). The reason for staying with the δ unit is because the differences measured in ^{13}C abundance among samples are very small compared to natural abundance of ^{13}C. For example, the $^{13}C/^{12}C$ ratio of the VPDB standard is 1.12372, and a δ = 0.1‰ corresponds to a change in $^{13}C/^{12}C$ of = 0.00012 producing a new ratio equal to 1.12384. However, physiological expression of ^{13}C abundance can only be done in terms of enrichment – that is in terms of the amount of ^{13}C in excess of natural ^{13}C. These expressions require a mole fraction measurement of ^{13}C.

The first transformation to a mole fraction that needs to be done is to convert the δ unit to the ratio of $^{13}C/^{12}C$ in the sample:

$$R = (\delta/1000 + 1)R_{std}$$

The ratio of $^{13}C/^{12}C$ is then converted to the atom fraction of ^{13}C abundance (F): $F = R\ (1+R)$ which correspond to $F = {}^{13}C / ({}^{13}C+{}^{12}C)$. As per the $^{13}C/^{12}C$ ratio, expressing ^{13}C abundance as $^{13}C/(^{13}C+^{12}C)$ still produces a number that is large compared to the difference in enrichment measured *among* samples with IRMS. Thus, a "δ" equivalent is used by subtracting natural abundance of ^{13}C (F_0) from each measured sample's abundance (F). This expression is

$$E = F - F_0$$

where E is the abundance of ^{13}C above natural abundance. Because the differences expressed by E are small, the values are expressed in ppm of excess ^{13}C. Note also that a sample that contains presence of administered exogenous compound could produce an E value either positive or negative, depending upon the abundance of the ^{13}C in the exogenous material. In general, most synthetically derived compounds are isotopically lighter than endogenously synthesized material and E is generally negative.

BRENNA
Correct, but the problem is solvable.

MATTHEWS
I want to make two points. One, better chromatography solves a host of problems, no question about that. Two, peaks that are buried under your peak are not going to be resolved by curve fitting or any other method whatsoever.

ISOSTER – Determination of the Origin of Hormones in Cattle

Angelika Preiss-Weigert[+1], Hildburg Fry[+1], Stefanie Banneke[+1], Bruno Le Bizec[+2], Wilhelm Schänzer[+3], Ed Houghton[+4] and Matthew Sharman[+5]

[+1] BfR, Berlin, D
[+2] LABERCA, ENV, Nantes, F
[+3] DSHS, Cologne, D
[+4] HFL, Fordham, UK
[+5] CSL, York, UK

The ISOSTER project is funded by the EC within the Growth programme as a part of the 5th framework programme. Four main partners (BfR, LABERCA, DSHS and CSL) are assisted by the Queens University in Belfast (QUB), UK, and by the TNO in Zeist, NL. Furthermore an added advantage is that the project involves three industrial partners: Thermo Finnigan, Bremen, D; GV Instruments, Manchester, UK; and DIONEX, Idstein, D.

The long term aim of the ISOSTER project is the creation of a tool for food control laboratories to allow them to control the administration of natural steroid hormones as growth promoting agents in food production.

The usual sampling locations of food control determine the choice of the matrix for analysis: as a consequence of this, methods applying GC-C-IRMS has to be developed for urine, feces, muscle, kidney, and fat samples. Additionally a major objective of this project is the validation of the GC-C-IRMS method for urine samples.

A further objective is the preparation of authentic samples derived from animals with a known history and kept under controlled conditions. In several animal trials, performed at the animal farm of the BfR in Berlin, samples from different categories of cattle (females and males, young animals and adults) have been collected. Thoroughly designed conceptual plans for the animal trials, taking into consideration the influences of feeding, should allow the variation of isotopic data of the steroids with and without administration of steroid hormones to be determined.

The aim of this project is to assess the effectiveness of the developed GC-C-IRMS methods for the differentiation between the endogenous and exogenous origin of steroids in cattle.

Isotope geochemists and chemists studying organic reaction mechanisms have studied isotope effects of chemical reactions extensively. The isotope effects are generally proportional to the square root difference between the two isotopes being studied. For example, the isotope effect of deuterium from protium is in the range of $^2H/^1H = [2/1]^{½} - 1 = 41\%$ or more, while that of $^{13}C/^{12}C = [13/12]^{½} - 1 = 4\%$ or less. The isotope effects seen in nature approximate these differences in mass alone. Although significantly greater natural differences will be found for hydrogen, due to its larger isotope effects, use of isotope effects to detect exogenously given metabolites has centered on measuring isotope differences of carbon because methods of measurement of physiological samples have been developed more fully. Isotope ratio mass spectrometry (IRMS) was developed in the 1930's for measurement of light element isotopes and saw immediate application to measurement of isotopes of carbon, nitrogen, oxygen, and hydrogen.

The hydrogen isotopes have always been the most problematic to measure, while carbon isotopes of organic compounds have been the simplest because the material to be measured only has to be purified and oxidized to CO_2 for measurement by IRMS. The only drawback is that purification and isolation of a metabolite in small amounts from a physiological sample is not a simple task. In 1978 Matthews and Hayes, demonstrated a new technique that eventually came to be known as gas chromatography-combustion-IRMS (GC-C-IRMS). The technique put a GC as the front end of the mass spectrometer and inserted an oxidation oven between the GC and an IRMS to oxidize all material eluting from the GC to CO_2. This technique took advantage of the reduction of noise by measuring all ^{13}C as CO_2 and measuring all samples against a reference CO_2 gas of known isotopic content. Since the initial 1978 report, a number of developments (e.g. introduction of fused silica GC columns) made commercial instruments feasible, and many laboratories around the world now have GC-C-IRMS instruments as their primary approach to IRMS measurement.

The GC-C-IRMS measurement for carbon measures the $^{13}CO_2/^{12}CO_2$ ratio for eluting compounds from the GC against a standard reference CO_2 gas of known ^{13}C content. Thus, the common values reported from the GC-C-IRMS are ^{13}C values in delta (δ) units in per mil (‰):

AUCHUS
To get rid of that little peak do you have to de-convolute or can you just do that with your single curve fitting?

BRENNA
Well, that is what you are doing. The word de-convolute is what is applied to what we are doing.

GEORGAKOPOULOS
If the software has the background subtraction algorithm, what is your opinion, is it useful to use and safe or not?

BRENNA
If you are thinking about a particular bit of software, I would not be able to address it specifically. All of the software has to subtract background; that is not optional. It cannot calculate an isotope ratio without subtracting background in some way.

BOWERS
Two points and see if you can synthesize these together. One, as you have gathered from this whole discussion the last couple of days, the sample matrix that we are dealing with contains a large number of substances, so the chances of having a really pure system are slim. Second, as you do this integration it is a serious issue to get de-convolated peaks. The problem I have is you are never going to standardize exactly between instruments, in particular, how this gets done. So what would your approach to this be? The summation issue is not going to go away, and you touched on the start and stop, which becomes even more important as you get overlapping things like that so where do we go from here?

BRENNA
An advantage to curve-fitting is that the start and stop points are not so important because they just specify a little more or less of the curve that is to be fit, and standardizing baselines and the possible overlaps is easier to handle. To answer the question in general, one would have to have of a general idea of what the range of expected chromatograms might be, including the typical contaminants.

BOWERS
Again, they are going to be sample specific, and so you are going to have to do something to deal with each sample.

Natural abundance, F_0, will vary among individuals based upon dietary ^{13}C differences, etc. Thus, instead of using a fixed standard value for F_0, it is adjusted for each measurement set. Using an F_0 value established for each person measured allows definition of ^{13}C enrichment above (or depletion below) natural abundance to be established individually. The question is how do we obtain a natural abundance measurement of the amount of ^{13}C (i.e. F_0) present in a particular metabolite in a particular sample for a particular subject? Any measurement we want to make in a sample assumes that exogenous material may be present and the metabolite to be measured will be different from natural abundance ^{13}C. One approach is to define a reference value of natural abundance for the population being measured. Doing so requires that we assess various variables that might affect the ^{13}C natural abundance. These variables include diet, sex, and race. Diet would include people living in different parts of the world and consuming diets for different areas and different natural variations in ^{13}C abundance. While reference values could be established, there will be significant uncertainty associated with them. Adding further is the requirement that every lab accurately and precisely standardize their reference ^{13}C measurements to assure agreement in baseline ^{13}C abundances. The net effect becomes a diminished ability to detect changes in ^{13}C abundance due to increased windows of uncertainty in the reference ^{13}C values required to account for variability's.

An alternative approach is to take advantage of other metabolites that are generally present in the measurement of the test metabolite. Usually the synthetic pathways of the various metabolites that are measured have more than one route of synthesis, and a metabolite can be found that is typical, but unrelated to the test metabolite. The ^{13}C abundance of this typical, but unrelated metabolite can be used to define the reference ^{13}C abundance for each person measured. The added value of this approach is that many errors disappear. Errors in knowing exactly the reference $^{13}CO_2$ gas used to calibrate the instrument largely disappear because the test metabolite and the reference metabolite are measured with the same error offset, and the offsets disappear upon subtraction of one from the other. Likewise dietary and race differences are anticipated to affect equally the different metabolites with respect to ^{13}C abundance. Again these differences disappear when the ^{13}C abundance of the reference metabolite is subtracted from the test metabolite.

In summary, GC-C-IRMS measurement of isotopic abundance is a powerful tool to differentiate exogenously administered compounds that exist endogenously. The principal power of the technique is seen when measurements are expressed not as the archaic δ nomenclature, but as the fractional abundance of the measured isotope from natural abundance. This measurement is especially powerful when it can be combined with a determination of natural abundance of a metabolite in the sample that is related to the test metabolite, but is unaffected by the exogenous administration of the suspected drug. Although ^{13}C measurements are most commonly used, greater power could be found using ^{2}H isotope measurements because of the greater differences in ^{2}H isotope abundance compared to ^{13}C. Possibly the most powerful technique comes from combined ^{2}H and ^{13}C abundance measurements.

Question & Answer Discussion

AUCHUS
Is there anything unique about the Tert-Butyl Dimethyl Silyl Group (TBDMS) is it because it has the tert-butyl group? If you just put trimethyl silyl group (TMS) on, do you still see this?

MATTHEWS
We stopped at that point and said this is ridiculous. From a chromatographic point of view, the TMS derivative is not as nice and clean and stable, and is a more difficult, ugly thing to use, although it is a classic derivative. My anticipation is that you will see exactly the same thing. I think what happens is, one of the silicon carbon bonds never breaks. That forms the basis for the silicon carbide formation. If you look in the ceramics literature, you actually can find it. That silicon carbide is a nice ceramic, and it will do exactly what it does and describes. My presumption is that you find it with the TMS derivative as well.

BOWERS
If you read the literature, there is discussion about silicon actually poisoning the copper nickel catalyst that you use for combustions. Is that not a concern? Is this possibly an explanation for that poisoning? Or are the people who say that it is poison just not correct?

more sophistication to look carefully and recognizes a good curve shape". At any rate, I do not know to what degree this has been incorporated in to other software.

VERKOUTEREN
In your curve fitting you mention there are four parameters to the curve. Are those determined empirically or exactly how were the parameters decided upon? Are they variable from instrument to instrument or from sample to sample?

BRENNA
They are variable from run to run. You can think the four parameters as exactly analogous to the two parameters you use for a calibration curve. With a linear regression, calculation of the slope and intercept is possible in closed mathematical form; so you can say the slope equals something and the intercept equals something else. Most curves cannot be modeled in closed form, as it turns out, so we use something called a Marquardt-Levenburg algorithm. It searches parameter space and finds a global minimum.

AUCHUS
You would think that if you have potential problems of small overlapping peaks, particularly if they are buried in your tails, that there might be some advantage of slicing the center cut of the peak. If you use your curve fitting you have to use the whole curve. If you use summation, I would think, that you could select what part of that curve you could sum. Where you did those experiments with the mixture, where you had low abundance that overlapped, a little bit, did you notice any difference in doing it those two ways?

BRENNA
First, using slices will not work because ^{13}C comes out a little bit earlier than ^{12}C, and so you have to integrate the whole peak to obtain an accurate isotope ratio. It is not like GCMS.

The rest of the paper is where we address the other cases, and evaluated a 1-10 and a 10-1 ratio of peak areas. If you have a 10-1 ratio, where you have a big peak first and a little one on the tail, curve fitting nicely recovers the big peak; I would not use the isotope ratio from little peak. The reason is that there is too much uncertainty in the shape of the baseline. The baseline in continuous flow is what drives everything.

AUCHUS
To get rid of that little peak do you have to de-convolute or can you just do that with your single curve fitting?

BRENNA
Well, that is what you are doing. The word de-convolute is what is applied to what we are doing.

GEORGAKOPOULOS
If the software has the background subtraction algorithm, what is your opinion, is it useful to use and safe or not?

BRENNA
If you are thinking about a particular bit of software, I would not be able to address it specifically. All of the software has to subtract background; that is not optional. It cannot calculate an isotope ratio without subtracting background in some way.

BOWERS
Two points and see if you can synthesize these together. One, as you have gathered from this whole discussion the last couple of days, the sample matrix that we are dealing with contains a large number of substances, so the chances of having a really pure system are slim. Second, as you do this integration it is a serious issue to get de-convolated peaks. The problem I have is you are never going to standardize exactly between instruments, in particular, how this gets done. So what would your approach to this be? The summation issue is not going to go away, and you touched on the start and stop, which becomes even more important as you get overlapping things like that so where do we go from here?

BRENNA
An advantage to curve-fitting is that the start and stop points are not so important because they just specify a little more or less of the curve that is to be fit, and standardizing baselines and the possible overlaps is easier to handle. To answer the question in general, one would have to have of a general idea of what the range of expected chromatograms might be, including the typical contaminants.

BOWERS
Again, they are going to be sample specific, and so you are going to have to do something to deal with each sample.

Natural abundance, F_0, will vary among individuals based upon dietary ^{13}C differences, etc. Thus, instead of using a fixed standard value for F_0, it is adjusted for each measurement set. Using an F_0 value established for each person measured allows definition of ^{13}C enrichment above (or depletion below) natural abundance to be established individually. The question is how do we obtain a natural abundance measurement of the amount of ^{13}C (i.e. F_0) present in a particular metabolite in a particular sample for a particular subject? Any measurement we want to make in a sample assumes that exogenous material may be present and the metabolite to be measured will be different from natural abundance ^{13}C. One approach is to define a reference value of natural abundance for the population being measured. Doing so requires that we assess various variables that might affect the ^{13}C natural abundance. These variables include diet, sex, and race. Diet would include people living in different parts of the world and consuming diets for different areas and different natural variations in ^{13}C abundance. While reference values could be established, there will be significant uncertainty associated with them. Adding further is the requirement that every lab accurately and precisely standardize their reference ^{13}C measurements to assure agreement in baseline ^{13}C abundances. The net effect becomes a diminished ability to detect changes in ^{13}C abundance due to increased windows of uncertainty in the reference ^{13}C values required to account for variability's.

An alternative approach is to take advantage of other metabolites that are generally present in the measurement of the test metabolite. Usually the synthetic pathways of the various metabolites that are measured have more than one route of synthesis, and a metabolite can be found that is typical, but unrelated to the test metabolite. The ^{13}C abundance of this typical, but unrelated metabolite can be used to define the reference ^{13}C abundance for each person measured. The added value of this approach is that many errors disappear. Errors in knowing exactly the reference $^{13}CO_2$ gas used to calibrate the instrument largely disappear because the test metabolite and the reference metabolite are measured with the same error offset, and the offsets disappear upon subtraction of one from the other. Likewise dietary and race differences are anticipated to affect equally the different metabolites with respect to ^{13}C abundance. Again these differences disappear when the ^{13}C abundance of the reference metabolite is subtracted from the test metabolite.

more dilute samples studied will have a negative excess of C13 over the standard. While this may be disconcerting to the reader, in any biological experiment what is really important is the difference in C13 concentrations in different compounds ..." *[emphasis in original]. A. O. C. Nier, In: Preparation and Measurement of Isotopic Tracers, 1946. Wilson, Nier, Riemann, Eds., p 28.*

2. B. F. Murphey, Phys. Rev. 1947, 72, 834–837.
3. K. J. Goodman and J. T. Brenna, Anal. Chem. 1994, 66(8) 1294-1301
4. K. J Goodman and J. T. Brenna, J. Chromatog A 1995, *689*, 63-68

Question & Answer Discussion

AGUILERA
Is Prof. Brenna looking at the curve fitting or using the summation?

BRENNA
Summation gave us errors but curve fitting did not, so curve fitting worked out nicely. If you have an overlap and you really, really, really, want to work out the isotope ratios, for instance you have a very valuable sample and you have just run it and there is no more, then overlaps can be handled with reasonable certainty.

MATTHEWS
When you say errors you mean inaccuracies?

BRENNA
Yes, an inaccuracy or bias.

AGUILERA
Do you know how many companies used the curve fitting approach?

BRENNA
No, these are all our algorithms. I do not know who has incorporated curve-fitting into their software. There has been talk over the years about incorporating it into software. It is not quite as straight forward as in the summation case because your curve shapes depend on your chromatography. I have some sympathy for the instrument manufacturers, in that they are trying to develop a machine that anybody can use for any curve shape. You get a number for any curve shape, but it takes a little bit

MATTHEWS
There are worse poisons. Using a fluoro derivative, like a heptafluoro butyryl derivative, produces a wonderful poison called hydrogen fluoride. This eats away at the oxidation tube itself. But amazingly enough people actually use that derivative and use it fairly effectively. The silicon derivatives degrade, which I think is a better word than poison, so what they do is they lay down a combination of silicon dioxide, and in this case, silicon carbide, which is going to eventually coat and reduce the active surface area. Most practitioners in the field say that if I am going to have to change one of these tubes ever couple of months, then it is not so bad. I don't think anyone has seen these reductions of efficiencies occur overnight. They occur slowly, and it doesn't really reduce the lifetime of the tube significantly.

Session 2:

Application of GC-C-IRMS to Doping Control

Chair:

Larry Bowers

dataset obtained from one vendor's IRMS and processed either with that vendor's algorithm or another vendor's algorithm. The results are shown in Figure 2 for about 20 fatty acid methyl esters. The overall precision of these analysis was SD ($\delta^{13}C$) <0.40‰. However

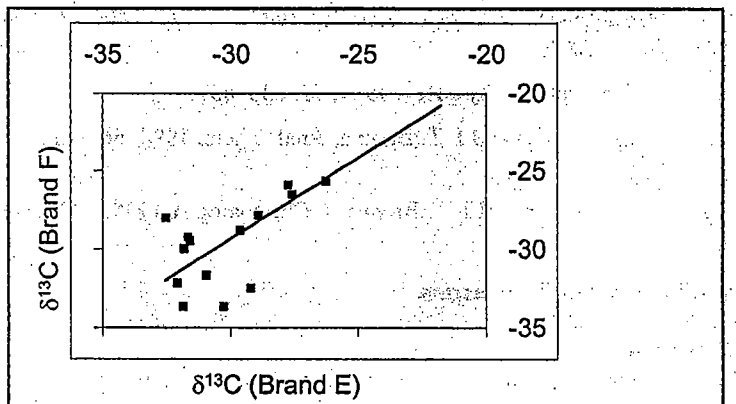


Figure 2. Raw data obtained for about 20 analytes on one vendor's IRMS and processed by two different algorithms. The average precision was SD($\delta^{13}C$)<0.40‰, but the difference in mean was considerably greater than this in most cases.

the root-mean-square difference in isotope ratios was about 2.1‰. It is important to emphasize that the raw data was identical; it was only the data reduction algorithms that differ.

We can conclude, overall, that the most reliable isotope ratio results are obtained when GC-C-IRMS peaks are strong and cleanly resolved from all other peaks. Though cross comparisons of algorithms are not common, insofar as they might be undertaken means isotope ratios may be unreliable for compounds with similar isotope ratios. Finally, these results demonstrate as dramatically as any, that *precision is no assurance of accuracy*, particularly in continuous flow IRMS. Good calibration standards are essential.

REFERENCES

1. "...it would seem highly advisable to always include in the published results the excess of tracer isotope ... above that found in some arbitrary standard. ... If ... ordinary chemical carbonate is used ... the normal average biological material will probably contain less C13 than does the laboratory standard and it and the

more dilute samples studied will have a negative excess of C13 over the standard. While this may be disconcerting to the reader, in any biological experiment what is really important is the difference in C13 concentrations in different compounds ..." *[emphasis in original]. A. O. C. Nier, In: Preparation and Measurement of Isotopic Tracers, 1946. Wilson, Nier, Riemann, Eds., p 28.*

2. B. F. Murphey, Phys. Rev. 1947, 72, 834–837.
3. K. J. Goodman and J. T. Brenna, Anal. Chem. 1994, 66(8) 1294-1301
4. K. J Goodman and J. T. Brenna, J. Chromatog A 1995, *689*, 63-68

Question & Answer Discussion

AGUILERA
Is Prof. Brenna looking at the curve fitting or using the summation?

BRENNA
Summation gave us errors but curve fitting did not, so curve fitting worked out nicely. If you have an overlap and you really, really, really, want to work out the isotope ratios, for instance you have a very valuable sample and you have just run it and there is no more, then overlaps can be handled with reasonable certainty.

MATTHEWS
When you say errors you mean inaccuracies?

BRENNA
Yes, an inaccuracy or bias.

AGUILERA
Do you know how many companies used the curve fitting approach?

BRENNA
No, these are all our algorithms. I do not know who has incorporated curve-fitting into their software. There has been talk over the years about incorporating it into software. It is not quite as straight forward as in the summation case because your curve shapes depend on your chromatography. I have some sympathy for the instrument manufacturers, in that they are trying to develop a machine that anybody can use for any curve shape. You get a number for any curve shape, but it takes a little bit

MATTHEWS
There are worse poisons. Using a fluoro derivative, like a heptafluoro butyryl derivative, produces a wonderful poison called hydrogen fluoride. This eats away at the oxidation tube itself. But amazingly enough people actually use that derivative and use it fairly effectively. The silicon derivatives degrade, which I think is a better word than poison, so what they do is they lay down a combination of silicon dioxide, and in this case, silicon carbide, which is going to eventually coat and reduce the active surface area. Most practitioners in the field say that if I am going to have to change one of these tubes ever couple of months, then it is not so bad. I don't think anyone has seen these reductions of efficiencies occur overnight. They occur slowly, and it doesn't really reduce the lifetime of the tube significantly.

Either summation or curve-fitting performs well for peaks represented by strong signals, flat and horizontal baselines, and excellent chromatographic resolution. Problems are encountered when peaks are close together or slightly overlapping, are of small signal, or appear on curved baselines (3). An example is shown in Fig 1. Two peaks of nearly identical $\delta^{13}C$ were progressively merged to yield varying degrees of overlap, and $\delta^{13}C$ calculated by summation or curve-fitting. Both algorithms produced excellent precision even in the presence of overlap as high as 40% valley. The summation algorithm means deviated from the known values, with the earlier eluting peaks apparently depleted, and the late eluting peak enriched. Curve-fitting is robust to overlap, maintaining good accuracy up to 40% valley. Superior performance has also been shown for curve-fitting in the case of low signal levels (4).

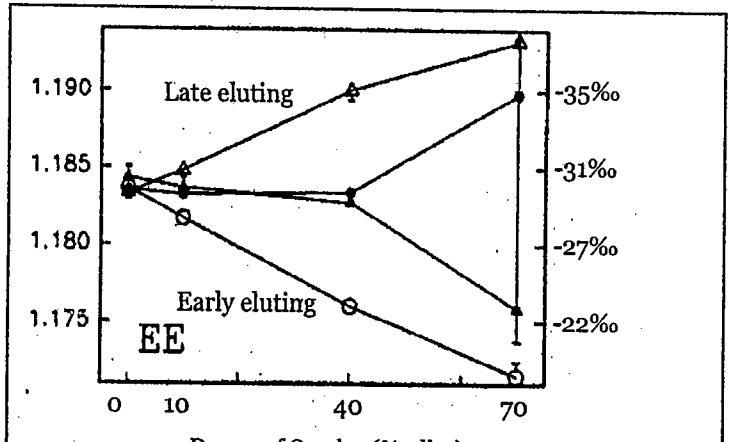


Figure 1. For data reduction by summation, the earlier eluting peak of a pair appears depleted while the later eluting peak appears enriched as a function of overlap. Curve-fitting does not suffer this problem until overlap is >40% valley. Left axis is R45 = I-45/I-44; right axis is $\delta^{13}C$. From Goodman and Brenna, Anal Chem 1994.

All algorithms do not result in the same isotope ratio when operating on the same data. We have recently observed a difference of about 0.7‰ when we mix summation and some forms of curve-fitting, though standardization against a single peak in a particular run removes this offset. A more dramatic example of this is found with a

$^{13}C/^{12}C$-Measurement of 17-Ketosteroids to detect Abuse of Androgenic Steroids in Sport

Ulrich Flenker, Ute Gunter, and Wilhelm Schänzer
German Sports University

The two 17-keto-steroids A and E are the most abundant metabolites of androgenic steroids in humans. This applies to the hormones T and DHT as well as to pro-hormones such as DHEA, Δ5-AED, and Δ4-AED.

Synthetic steroids are produced predominantly by partial synthesis from phytosterols such as diosgenine, stigmasterine or sitosterine. Diosgenine is extracted from *Dioscorea* species, stigmasterine and sitosterine are extracted from soy. Due to their photosynthetic pathway these plants show a characteristic signature of the $^{13}C/^{12}C$-ratio. In case of administration of synthetic steroids this isotopic signature can be tracked forward to urinary excreted metabolites. Up to now only values have been reported that are significantly depleted in ^{13}C *vs.* endogenous steroids.

To detect the presence of exogenous steroids that contribute to androgenic metabolism the $\delta^{13}C$-values of the target compounds (TCs) A and E are compared to those of urinary steroids which are unrelated to this pathway. Such endogenous reference compounds (ERCs) are pregnanediol and 11-hydroxyAndrosterone.

To cope with analytical error on the one hand and with biological variation on the other, we established reference ranges (n = 60, students of physical education) for the isotopic composition of the TCs and the ERCs and for the isotopic differences between TCs and ERCs.

Table 1 shows the 99.9% reference limits and the corresponding 90% confidence intervals. With respect to differences between A and corresponding ERCs $\delta^{13}C_{PDB}$ -values of more than about 2.4 per mil are incompatible with sole presence of endogenous steroids assuming ^{13}C-depletion of the synthetic material. The values for E are somewhat larger, which we attribute to small isotopic fractionation during metabolism.

Anyway an apparent isotopic difference between **E** and **A** might also be due to chromatographic overlap. Although true baseline separation between **E** and **A** was achieved, we also calculated reference values for the differences between the ERCs and **E** and **A** integrated jointly. We hereby intended to construct an additional robust and integrative parameter.

The analytical method briefly is as follows:

1. Sample Preparation
   (a) 10 ml urine
   (b) SPE purification
   (c) Hydrolysis (β-glucuronidase, *E. coli*)
   (d) HPLC-purification, 3 fractions
   - Column: MERCK LiChrospher 100 RP-18, 200 × 4 mm, 5 µm
   - Flow: 1 ml/min
   - Solvent: H_2O/CH_3CN 70:30 ↦ 0:100 within 20 min
   - Automatic collection after standard measurement
   - **T** acetate as reference standard

2. GC Conditions
   (a) Hewlett Packard 5890-II
   (b) Column: Macherey & Nagel Optima-δ-3, 20 m × 0.25 mm, 0.25 µm film
   (c) Carrier gas: Helium (quality 5.0, additional gas purification), constant pressure at 30 psi
   (d) Injection: Splitless at 60°C, 1-3 µl (depending on concentration)
   (e) Temperature: 30°C/min ↦ 265°C, 3°C/min ↦ 295°C

3. Combustion Interface
   - FINNIGAN-MAT GC-Combustion Interface II, customized:
     (a) Metal X-piece replaced by Y-press-fit connector
     (b) Backflush valve moved outside the GC
     (c) Connection to reactor drilled up to 0.5 mm, capillary almost in touch with Cu/Ni/Pt wires
     (d) Reactor elevated about 1.5 cm, **mandatory** for measurement of free steroids

Effects of Chromatographic Overlap on Uncertainty

Tom Brenna
Cornell University

Isotope ratio mass spectrometry (IRMS) is the method by which the most abundant isotopes of the organic elements are measured with the highest precision. The relevant elements are C, H, O, N, and S. The IRMS instrument is optimized to measure, for instance, CO_2, for $^{13}C/^{12}C$ and $^{18}O/^{16}O$ with highest sensitivity to produce the best counting statistics for highest precision. This optimization comes at the expense of flexibility; all analytes must be converted to CO_2 prior to admission to the IRMS. Measurement to measurement precision expressed as coefficient of variation is around 0.03%. To limit the redundancy in reporting of results, and to emphasize the practice of measuring isotope ratios *relative* to a standard, the PDB notation was introduced as first outlined by Nier (1), $\delta^{13}C$, as the difference in isotope ratio between an international standard carbonate and the sample, in parts per thousand.

The original method for introducing sample into the instrument is due to Murphey (2), and is called the "dual inlet", referring to the use of two volumes for CO_2 gas, one for sample, the other for standard. This method yields raw data that is approximately constant and isotope ratios are easy to calculate from the raw data. More recent methods are called "continuous flow", because helium gas sweeps the analyte into the ion source. GC-C-IRMS is the prototypical technique in this category. Data reduction, the conversion of raw data into isotope ratios, is not straightforward in this technique because the signal is constantly changing. The analyte peak must be defined, integrated, and background subtracted to obtain the raw isotope ratio, and this must all be done with reproducibility quoted above. Several algorithms exist for this, and the "summation" algorithm is probably the most widely used. Here, a peak start and stop are defined by some criteria related to signal slope, background points are determined, and subtracted. An alternative is "curve-fitting", whereby the signal is modeled by a mathematical function that has the shape of a skewed bell-shaped curve. At least eighty different functions have been used to model chromatographic peaks. An example is the exponentially-modified Gaussian, which is a mathematical convolution of an exponential decay curve and a Gaussian.

Either summation or curve-fitting performs well for peaks represented by strong signals, flat and horizontal baselines, and excellent chromatographic resolution. Problems are encountered when peaks are close together or slightly overlapping, are of small signal, or appear on curved baselines (3). An example is shown in Fig 1. Two peaks of nearly identical $\delta^{13}C$ were progressively merged to yield varying degrees of overlap, and $\delta^{13}C$ calculated by summation or curve-fitting. Both algorithms produced excellent precision even in the presence of overlap as high as 40% valley. The summation algorithm means deviated from the known values, with the earlier eluting peaks apparently depleted, and the late eluting peak enriched. Curve-fitting is robust to overlap, maintaining good accuracy up to 40% valley. Superior performance has also been shown for curve-fitting in the case of low signal levels (4).

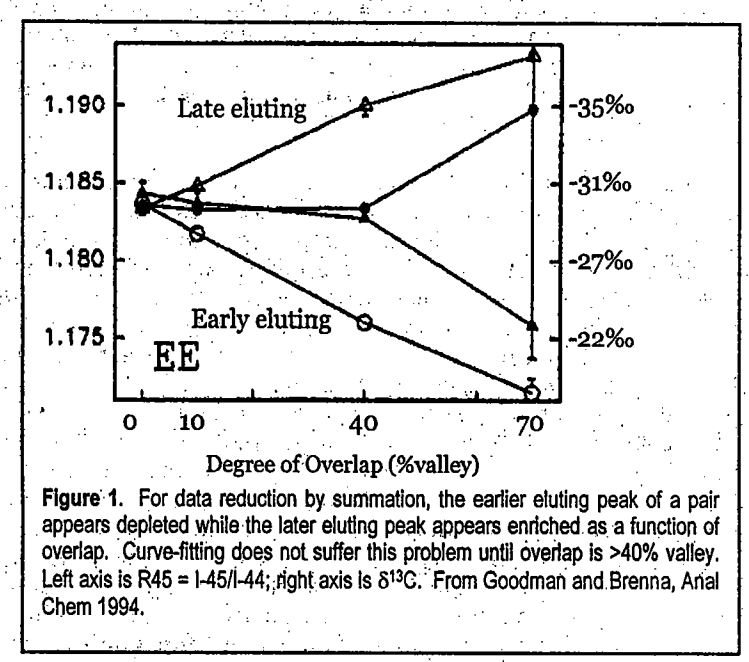


Figure 1. For data reduction by summation, the earlier eluting peak of a pair appears depleted while the later eluting peak appears enriched as a function of overlap. Curve-fitting does not suffer this problem until overlap is >40% valley. Left axis is R45 = I-45/I-44; right axis is $\delta^{13}C$. From Goodman and Brenna, Anal Chem 1994.

All algorithms do not result in the same isotope ratio when operating on the same data. We have recently observed a difference of about 0.7‰ when we mix summation and some forms of curve-fitting, though standardization against a single peak in a particular run removes this offset. A more dramatic example of this is found with a

$^{13}C/^{12}C$-Measurement of 17-Ketosteroids to detect Abuse of Androgenic Steroids in Sport

Ulrich Flenker, Ute Gunter, and Wilhelm Schänzer
German Sports University

The two 17-keto-steroids **A** and **E** are the most abundant metabolites of androgenic steroids in humans. This applies to the hormones **T** and DHT as well as to pro-hormones such as DHEA, Δ5-AED, and Δ4-AED.

Synthetic steroids are produced predominantly by partial synthesis from phytosterols such as diosgenine, stigmasterine or sitosterine. Diosgenine is extracted from *Dioscorea* species, stigmasterine and sitosterine are extracted from soy. Due to their photosynthetic pathway these plants show a characteristic signature of the $^{13}C/^{12}C$-ratio. In case of administration of synthetic steroids this isotopic signature can be tracked forward to urinary excreted metabolites. Up to now only values have been reported that are significantly depleted in ^{13}C *vs.* endogenous steroids.

To detect the presence of exogenous steroids that contribute to androgenic metabolism the $\delta^{13}C$-values of the target compounds (TCs) **A** and **E** are compared to those of urinary steroids which are unrelated to this pathway. Such endogenous reference compounds (ERCs) are pregnanediol and 11-hydroxyAndrosterone.

To cope with analytical error on the one hand and with biological variation on the other, we established reference ranges (n = 60, students of physical education) for the isotopic composition of the TCs and the ERCs and for the isotopic differences between TCs and ERCs.

Table 1 shows the 99.9% reference limits and the corresponding 90% confidence intervals. With respect to differences between **A** and corresponding ERCs $\delta^{13}C_{PDB}$ -values of more than about 2.4 per mil are incompatible with sole presence of endogenous steroids assuming ^{13}C-depletion of the synthetic material. The values for **E** are somewhat larger, which we attribute to small isotopic fractionation during metabolism.

programs you gravitate toward a mean. This is a fairly well known phenomenon. What I am trying to understand is the role you or NIST plays and to introduce this data management handling process into it. I am trying to figure out whether or not you just had a group of labs that worked hard, as you described, and did better over time. Therefore, the results got better, or how much of the contribution was because you were able to devise a central data management handling system.

VERKOUTEREN

The first inter-comparison was done with a very different set of labs. There were only maybe two labs that were common to both so the improvement in the results cannot be attributed to understanding the sample better. That was not it at all. Then, because we asked for measurement results rather than $\delta^{13}C$ results, they could not have possibly gotten better by knowing anything about those materials. Each laboratory has their own working reference gas, so they have their own delta measurements. In fact the composition of the working reference gas is totally immaterial in the inter-comparison because we did not reference to the working reference gas, but we referenced to another sample in the sequence. We only use the working reference gas to correct for drift, but the sample and standard all go through the same inlet. If there are any kinds of differences, in fractionation between inlets, they are corrected for in that way. Plus the samples were all of the same size, so they are all size matched. That was another reason why this inter- comparison may have had better results...it was more rigorously designed.

What you are trying to do is extremely challenging. You will find the first time around will be very discouraging. You will find you are way, way off having any kind of harmony, which is totally natural. It is going to take some time and resources, and it is going to require a lot of commitment to be able to reach harmony. I do not think it has really been done to this scale anywhere worldwide, and I am willing to help as much I can. It is going to take everyone's cooperation, everyone's knowledge and trust, to be able to achieve that. It is doable. It is just going to take a lot of effort.

(e) Reduction furnace unmounted
(f) Nafion water trap replaced by cryotrap (Dewar, acetone/dry ice)

All compounds are measured in free form. Provided the GC-C system is in good condition this does not show any significant disadvantages compared to acetylation or silylation. On the other hand this circumvents possible problems with incomplete reaction and with blurring of isotopic signatures.

Table 1: Reference limits (99.9%) and corresponding confidence intervals (CI, 90%) for relevant $\delta^{13}C$-values. A: Androsterone; E: etiocholanolone; EA: E and A, common integration; PD: pregnanediol; 11OHA: 11-hydroxyAndrosterone.

	Reference Limits		
$\Delta\delta^{13}C$	Lower	Upper	CI
PD - E	-1.01	2.88	0.35
PD - A	-2.22	1.89	0.37
PD - EA	-1.70	2.27	0.36
11OHA - E	-1.49	3.73	0.49
11OHA - A	-1.84	1.83	0.34
11OHA - EA	-1.46	2.36	0.36

Applying GC-C-IRMS to samples that exhibited unusual steroid profiles, we were able to show presence of exogenous steroids as well as presumed absence.

Table 2 shows values of a sample reported negative: Especially the Testosterone/Epitestosterone (T/E) ratio and the T concentration are unusually high. Although these parameters might indicate T administration, $^{13}C/^{12}C$-ratios suggest physiological reasons for this observation.

Table 2: Sample 30638/2002: T/E = 8.1, A/T = 27, c_T = 157 ng/ml. Sample reported negative

Compound (n = 1)	$\delta^{13}C_{PDB}$
A	-23.0
E	-24.1
11OHA	-24.3

Table 3 conversely describes a sample that is mostly unsuspicious with respect to T. Yet the A/E ratio is unusual low, might indicate application of steroids that preferably are metabolized to E. Indeed isotopic data clearly show depleted $\delta^{13}C$-values. E is affected more than A. The whole pattern can be explained by administration of Δ5-pro-hormones.

Table 3: Sample 1145/2003: T/E = 4.9, A/E = 0.22, c_T = 5.3 ng/ml. Sample reported positive

Compound (n = 3)	$\delta^{13}C_{PDB}$
A	-26.4
E	-29.7
11OHA	-20.1
PD	-21.4

Question & Answer Discussion

AGUILERA
Do you know what amount of urine they used? You never used less than 10ml?

Question & Answer Discussion

CATLIN
The experiment was very interesting that Dr. Verkouteren described, where a group of labs got together and did measurements, and then sent data through the algorithm that he described. He was pleased to find no outliers. Was that in the second trial? He must have specified outliers in the first go around or did the algorithm remove them all?

VERKOUTEREN
I only got $\delta^{13}C$ and $\delta^{18}O$ results for the CO_2. I had no information about what algorithms were used, I had no basis for throwing out any outliers and all the data you saw was all the data I collected for the first trial. For the second trial, I did not collect $\delta^{13}C$. I only collected the $\delta^{45}CO_2$ and $\delta^{46}CO_2$ and then a lot of information about the discretionary factors. Once I went through the algorithm and all the data handling in a very consistent way, I only had one outlier from a 100 results. That particular outlier had a procedural explanation. That was for VSMOW, the $\delta^{18}O$ of VSMOW. That particular lab used much too little water in the equilibration with a CO_2, so they were not following the best practice. That was identified and information transmitted back to them. They learned that fact from this inter-comparison.

Something else I always do is to make the reported inter-comparison results anonymous, so they are not linked with any particular lab. I respect the privacy of the lab, I do not want them to be embarrassed in anyway, and so I need enough labs so that the pool of data is large. Each lab gets to know who they are, of course, privately so they know what to then work on.

There is a NIST Report that came out on this, which the labs have had for about a year now. I get comments back when, after the labs find that, "oh my gosh, I did something wrong". They would like me to post-correct their data, but I go through that in the beginning. If I find something in the database that they report that just does not seem right, I will immediately come back and say, "is this right, is it a typo, or what is going on?" They have to verify to me that these particular values that they have reported are correct. At that point, they are hardened into stone, and then I go forth with the data evaluation.

CATLIN
This group does studies like that but not necessarily on carbon isotope ratio. We get a group of labs who participate in some sort of round robin, and we have seen over many, many years that if you participate in these PT

programs you gravitate toward a mean. This is a fairly well known phenomenon. What I am trying to understand is the role you or NIST plays and to introduce this data management handling process into it. I am trying to figure out whether or not you just had a group of labs that worked hard, as you described, and did better over time. Therefore, the results got better, or how much of the contribution was because you were able to devise a central data management handling system.

VERKOUTEREN

The first inter-comparison was done with a very different set of labs. There were only maybe two labs that were common to both so the improvement in the results cannot be attributed to understanding the sample better. That was not it at all. Then, because we asked for measurement results rather than $\delta^{13}C$ results, they could not have possibly gotten better by knowing anything about those materials. Each laboratory has their own working reference gas, so they have their own delta measurements. In fact the composition of the working reference gas is totally immaterial in the inter-comparison because we did not reference to the working reference gas, but we referenced to another sample in the sequence. We only use the working reference gas to correct for drift, but the sample and standard all go through the same inlet. If there are any kinds of differences, in fractionation between inlets, they are corrected for in that way. Plus the samples were all of the same size, so they are all size matched. That was another reason why this inter- comparison may have had better results…it was more rigorously designed.

What you are trying to do is extremely challenging. You will find the first time around will be very discouraging. You will find you are way, way off having any kind of harmony, which is totally natural. It is going to take some time and resources, and it is going to require a lot of commitment to be able to reach harmony. I do not think it has really been done to this scale anywhere worldwide, and I am willing to help as much I can. It is going to take everyone's cooperation, everyone's knowledge and trust, to be able to achieve that. It is doable. It is just going to take a lot of effort.

(e) Reduction furnace unmounted
(f) Nafion water trap replaced by cryotrap (Dewar, acetone/dry ice)

All compounds are measured in free form. Provided the GC-C system is in good condition this does not show any significant disadvantages compared to acetylation or silylation. On the other hand this circumvents possible problems with incomplete reaction and with blurring of isotopic signatures.

Table 1: Reference limits (99.9%) and corresponding confidence intervals (CI, 90%) for relevant $\delta^{13}C$-values. A: Androsterone; E: etiocholanolone; EA: E and A, common integration; PD: pregnanediol; 11OHA: 11-hydroxyAndrosterone.

	Reference Limits		
$\Delta\delta^{13}C$	Lower	Upper	CI
PD - E	-1.01	2.88	0.35
PD - A	-2.22	1.89	0.37
PD - EA	-1.70	2.27	0.36
11OHA - E	-1.49	3.73	0.49
11OHA - A	-1.84	1.83	0.34
11OHA - EA	-1.46	2.36	0.36

Applying GC-C-IRMS to samples that exhibited unusual steroid profiles, we were able to show presence of exogenous steroids as well as presumed absence.

RECOMMENDATIONS

The following recommendations are offered to help achieve the goal of international harmonization in GC-C-IRMS performance and results:

1. Identify specific analytical targets and acceptable limits of measurement uncertainty that are based on tolerable risks of false negative and false positive results.
2. Develop a suitable urine RMs, covering the range of observed steroid isotopic values, and assign documented isotopic values and uncertainties that are traceable to VPDB.
3. Develop performance-based standards to assess data quality through intercomparisons and proficiency tests.
4. In comparing two steroid measurements for a significant difference ($\Delta\delta$), these measurements should be performed under identical conditions and referenced to a RM to establish traceability to VPDB. Standard uncertainties of these two measurements can be used in a two-sided normal test (e.g., Natrella, 1963, Section 3-3.1.3) to determine whether the mean results are statistically distinct at a specified confidence level.

REFERENCES

1. Craig, H. (1957) *Geochim. Cosmochim. Acta* **12**, 133-149.
2. Eurochem/CITAC (2000) Guide to Quantifying Uncertainty in Analytical Measurement
3. Hut, G. (1987) Report of IAEA Consultants' group meeting on stable isotope reference samples
4. ISO-BIPM (1993) Guide to the Expression of Uncertainty in Measurement
5. ISO/IEC 17025 (1999) Requirements for the Competence of Calibration and Testing Laboratories
6. ISO-VIM (1993) International Vocabulary of Basic and General Terms in Metrology
7. Natrella, M. G. (1963) Experimental Statistics, NBS Handbook 91 (Reprinted 1966)
8. NIST SP260-149 (2003) Value Assignments and Uncertainty Estimation of Selected Light Stable Isotope Materials

THEVIS
I think it was 2ml up to 20 ml. The average was 10 ml. So if we look up our standard operations procedures, it actually depends on the density and the amount of urine that was actually available. The average is approximately 10ml.

AGUILERA
Ten milliliters of urine is just about 20,000 ng of andro/etio. If you use an HPLC purification, do you know if there were some fractionation? When you do purification by HPLC, it is easy to have isotopic fractionation.

THEVIS
That is the reason why the fractions are pretty broad. As you saw the fractions were not tiny, and you could observe this phenomenon if they were smaller. That is why the fraction is about 2 minutes although the peak width is only 20 seconds, just for example. We need these high amounts because we focus our instrument to maximum linearity, so we sacrifice the sensitivity. So we try to compensate these things.

KAZLAUSKAS
You mentioned that you do a screening first without HPLC. Do you do the HPLC only for confirmation?

THEVIS
That is right.

HOUGHTON
How do you account for variation in the difference between ERC and A/E? You got that slight difference of 1, how do you account for that?

THEVIS
It is a well known phenomenon that you will have this difference. It occurs in basically every sample. So we didn't find that it was only with our reference population but basically every sample that we analyzed. That the E delta values are different by approximately 1 compared to A.

HOUGHTON
The enzyme process seems to be discriminating between the two stable isotopes.

BOWERS
You said you swapped out the Nafion water trap to the Cryotrap. Was there some reason for that specifically?

THEVIS
The Cryotrap was much more reliable than the Nafion trap. We had serious problems with the membrane. He said he had to exchange it every now and then. So he went over to use the Cryotrap which is easily regenerated by heating with a heat gun for a couple of minutes, and it was up and running again.

While the example above deals with dual-inlet IRMS rather than GC-C-IRMS, these techniques share most components in the TMP (Fig.1). For doping control, the differences consist in the steps of sampling, stabilization, and chemical separation/purification. Admittedly, these steps most likely contain the greatest sources of measurement uncertainty, but the construct of the TMP allows the uncertainty budget to be expanded to account for these sources. Table 1 shows such a measurement traceability chain and estimated uncertainty budget.

Measurement Chain – Sample	Link in chain	Documentation	$\delta^{13}C$ value assignment	Uncertainty of Measurement Link (u_c)	Total Uncertainty to VDPB (u_c)
Matrix RM	5	Lab Report	Measurand	0.06 ‰ (est.)	0.12 ‰ (est.)
RM 8562 CO_2	4	TBD	TBD	0.10 ‰ (est.)	0.11 ‰ (est.)
NBS 19 limestone	3	NIST SP260-149 (2003)	-3.76 ‰	0.04 ‰	0.04 ‰
VPDB	2	Hut (1987)	+1.95 ‰	exact	0
SI	1	Craig (1957)	R = 0.0112372	0.0000300	(2.7 ‰)

Table 1: Traceability Chain and Estimated Uncertainty Budget for GC-C-IRMS Measurements

This chain of comparisons with traceability to VPDB presumes the development and use of a steroid isotope RM in a urine matrix with an uncertainty assigned through exhaustive type A and type B evaluations. Here, this combined uncertainty is estimated as 0.10 ‰, which reflects the anticipated uncertainty in operationally dependent isotope measurement comparisons between the steroid-specific CO_2 and a VPDB-traceable standard CO_2. Consequently, the total uncertainty of a sample measurement referenced to VPDB, with uncertainties propagated down the traceability chain by root-of-summed-variance, is estimated as 0.12 ‰. Traceability is commonly not extended to the Systeme International (SI) for delta measurements since this extra comparison (Link 1) introduces a large uncertainty that is unimportant in most applications.

RECOMMENDATIONS

The following recommendations are offered to help achieve the goal of international harmonization in GC-C-IRMS performance and results:

1. Identify specific analytical targets and acceptable limits of measurement uncertainty that are based on tolerable risks of false negative and false positive results.
2. Develop a suitable urine RMs, covering the range of observed steroid isotopic values, and assign documented isotopic values and uncertainties that are traceable to VPDB.
3. Develop performance-based standards to assess data quality through intercomparisons and proficiency tests.
4. In comparing two steroid measurements for a significant difference ($\Delta\delta$), these measurements should be performed under identical conditions and referenced to a RM to establish traceability to VPDB. Standard uncertainties of these two measurements can be used in a two-sided normal test (e.g., Natrella, 1963, Section 3-3.1.3) to determine whether the mean results are statistically distinct at a specified confidence level.

REFERENCES

1. Craig, H. (1957) *Geochim. Cosmochim. Acta* **12**, 133-149.
2. Eurochem/CITAC (2000) Guide to Quantifying Uncertainty in Analytical Measurement
3. Hut, G. (1987) Report of IAEA Consultants' group meeting on stable isotope reference samples
4. ISO-BIPM (1993) Guide to the Expression of Uncertainty in Measurement
5. ISO/IEC 17025 (1999) Requirements for the Competence of Calibration and Testing Laboratories
6. ISO-VIM (1993) International Vocabulary of Basic and General Terms in Metrology
7. Natrella, M. G. (1963) Experimental Statistics, NBS Handbook 91 (Reprinted 1966)
8. NIST SP260-149 (2003) Value Assignments and Uncertainty Estimation of Selected Light Stable Isotope Materials

THEVIS
I think it was 2ml up to 20 ml. The average was 10 ml. So if we look up our standard operations procedures, it actually depends on the density and the amount of urine that was actually available. The average is approximately 10ml.

AGUILERA
Ten milliliters of urine is just about 20,000 ng of andro/etio. If you use an HPLC purification, do you know if there were some fractionation? When you do purification by HPLC, it is easy to have isotopic fractionation.

THEVIS
That is the reason why the fractions are pretty broad. As you saw the fractions were not tiny, and you could observe this phenomenon if they were smaller. That is why the fraction is about 2 minutes although the peak width is only 20 seconds, just for example. We need these high amounts because we focus our instrument to maximum linearity, so we sacrifice the sensitivity. So we try to compensate these things.

KAZLAUSKAS
You mentioned that you do a screening first without HPLC. Do you do the HPLC only for confirmation?

THEVIS
That is right.

HOUGHTON
How do you account for variation in the difference between ERC and A/E? You got that slight difference of 1, how do you account for that?

THEVIS
It is a well known phenomenon that you will have this difference. It occurs in basically every sample. So we didn't find that it was only with our reference population but basically every sample that we analyzed. That the E delta values are different by approximately 1 compared to A.

underestimated, by about a factor of ten or more, the variation in measurement results observed across the laboratories. This suggested strongly that variations in the TMP used in each laboratory were responsible for the observed dispersion in results, and that another strategy was needed to enable better reproducibility of results and allow precise and accurate assignments of delta values for the RMs.

The Light Stable Isotope Working Group, an international panel of experts convened by the IAEA, designed an exercise for the isotopes of carbon and oxygen in nine RMs. This exercise was performed by a small number of laboratories, selected on the basis of their experience with carbonate and water sample preparations, and with an attempt to represent equally the variety of IRMS instruments. Standard chemical preparation procedures and sample measurement procedures, determined as "best practices," were mandatory, as were the documentation and reporting of non-mandatory (discretionary) procedures. Additionally, the raw measurement data for all measurements was reported. The measurement data was centrally processed using internationally accepted procedures so that no variations could enter during this stage of the TMP. Lastly, repeatability and consistency of replicated measurements reported in each laboratory formed the basis for analytical performance metrics. These metrics were used to assess the Type A uncertainty of each result relative to the Type B uncertainty estimated by the interlaboratory variation of mean results. When Type A uncertainty of any datum exceeded 0.2 ‰, which was about the upper limit of observed Type B uncertainty for the most challenging RM, that datum was excluded from the mean. The outcome of this exercise was that of 100 results calculated across the laboratories, there was only one 2-s outlier, and that upon inspection of discretionary procedures had an obvious explanation. Intra-laboratory repeatability matched relatively well with inter-laboratory reproducibility, and the value assignments resulting from the exercise were precise, technically sound, and consistent with a consensus mean of globally equitable data.

GC-C-IRMS Research at UCLA

Don Catlin, M.D.
University of California, Los Angeles

Our group became interested in carbon isotope ratio methodologies when Daniel Fraisse, Ph.D., visited UCLA in 1986. Fraisse described that there is a measurable difference in ^{13}C content between natural vanillin, which is expensive, and synthetic vanillin, which is inexpensive and often sold in place of natural vanillin, and that his institution in France had a GC-C-IRMS facility where such vanillin analyses were done. We wondered if there might also be a measurable difference between pharmaceutical and endogenous urinary T. By 1990, Southan et al[1] presented an abstract at a meeting in Barcelona that confirmed this possibility. When Fraisse's collaborators Becchi and Aguilera et al[2] reported that in urine collected before and after T administration, the $\delta^{13}C$ ("delta values") of urinary T and metabolites were significantly lower after T administration and that the delta values of metabolic precursors of T were unchanged, we began to collaborate with them.

Initially Aguilera joined our group but continued to work in France because we did not have an isotope ratio mass spectrometer (IRMS). It took three more years and several papers[2,3,4] to convince sport funding agencies in the U.S. that the method was sound and could ultimately resolve some of the difficulties with interpretation of T/E ratios that exceed 6. In due course we have verified and validated the diol method in our laboratory, and reported delta values in 73 healthy control subjects.[5,7] We believe it is possible to differentiate between urine samples from athletes with the naturally elevated T/E syndrome and athletes who have used T.[7] Although the latter manuscript cannot prove the concept, because we do not yet have good ways to define what the syndrome is, it does suggest that eventually this objective might be obtained.

Later we extended the application of the method to A and E[6] and to epi-T.[8] For T/E cases and for general screening we greatly prefer the Androsterone/E assay, even though it does not include an endogenous reference standard, because it is substantially shorter and simpler than the diol assay. Further the low concentrations of diols in some male

urines and most female urines greatly limit the applicability of the diol assay.

The epi-T method is sound and relatively robust. The mean delta value for 43 healthy males controls was -23.8 $^{0}/_{00}$ which is similar to other urinary steroids. We do not have a reference group for females. It is difficult to fully validate the method in man because epi-T is not approved for human use in the U.S. Therefore we cannot administer epi-T unless we obtain approval from the U.S. Food and Drug Administration—an onerous and expensive procedure. Nevertheless we have encountered one subject with a very high urine epi-T level (1,176 ng/mL). The delta value of this person's epi-T (-32.6 $^{0}/_{00}$) was very low as expected. We hope to obtain FDA approval for epi-T studies in the near future.

We have now extended the method to AED. Six male subjects, all of whom denied participation in competitive weightlifting, a history of cardiopulmonary disease, malignancy, prostate disease, major psychiatric disease, substance abuse, or use of any medication known to affect steroid hormone or binding protein levels, were recruited. Subjects were also excluded if they reported prior use of AED or androgenic anabolic steroids. All subjects were required to have normal serum T and creatinine levels, and normal liver function tests. All participants gave written informed consent and all urine was collected for three consecutive days. Days 1 and 3 were control days and no drug was administered. On day 2 the subjects received 50 mg of AED per os and all subsequent urine was collected for the ensuing 24 hours.

Chemistry (CITAC) (2000) (http://www.measurementuncertainty.org and at http://physics.nist.gov/cuu/Uncertainty).

PRACTICAL ISSUES: A RELEVANT EXAMPLE

Reference materials (RMs) are considered one of the critical components in the assurance of analytical quality, allowing a measure of accuracy within laboratories and an assessment of interlaboratory reproducibility and harmony. For applications important to national and international interests, reliable RMs have been developed and certified by various National Measurement Institutes (NMI) and National Reference Laboratories.

The NIST, as the NMI of the United States, has worked with the IAEA and other organizations to provide a limited number of isotope RMs, mainly pure compounds, for use to test analytical procedures, calibrate instruments, and to enable measurement traceability to internationally accepted reference scales. Value assignments for most of these isotopic RMs were determined through international comparison exercises. The first exercises were "result intercomparisons" where the materials were distributed to all laboratories interested in participating. Those laboratories that responded simply reported a measurement result and uncertainty for each material. In all these exercises, the uncertainty reported from any laboratory grossly

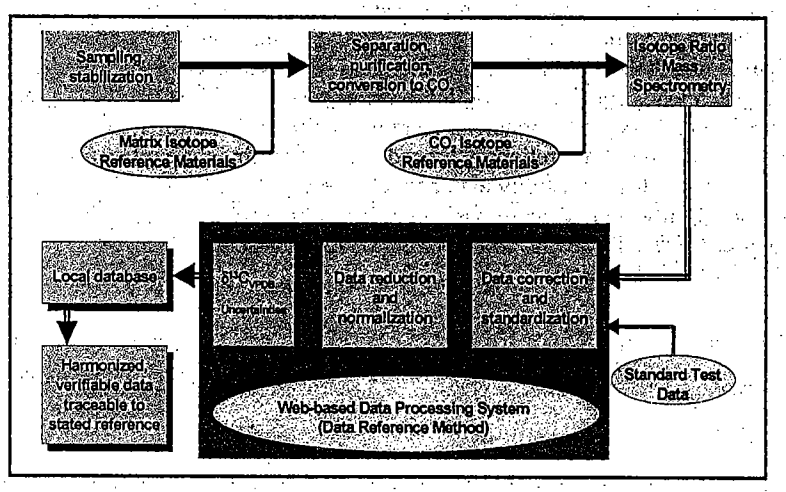


Figure 1. The GC-C-IRMS Total Measurement Process (TMP). A schematic depiction of the flow of chemical and digital information from sampling to reporting of results. The TMP considers every step in the analytical train, and shows the use of quality assurance tools (designated by ovals) that test the fidelity of the analytical steps "downstream" from their input.

underestimated, by about a factor of ten or more, the variation in measurement results observed across the laboratories. This suggested strongly that variations in the TMP used in each laboratory were responsible for the observed dispersion in results, and that another strategy was needed to enable better reproducibility of results and allow precise and accurate assignments of delta values for the RMs.

The Light Stable Isotope Working Group, an international panel of experts convened by the IAEA, designed an exercise for the isotopes of carbon and oxygen in nine RMs. This exercise was performed by a small number of laboratories, selected on the basis of their experience with carbonate and water sample preparations, and with an attempt to represent equally the variety of IRMS instruments. Standard chemical preparation procedures and sample measurement procedures, determined as "best practices," were mandatory, as were the documentation and reporting of non-mandatory (discretionary) procedures. Additionally, the raw measurement data for all measurements was reported. The measurement data was centrally processed using internationally accepted procedures so that no variations could enter during this stage of the TMP. Lastly, repeatability and consistency of replicated measurements reported in each laboratory formed the basis for analytical performance metrics. These metrics were used to assess the Type A uncertainty of each result relative to the Type B uncertainty estimated by the interlaboratory variation of mean results. When Type A uncertainty of any datum exceeded 0.2 ‰, which was about the upper limit of observed Type B uncertainty for the most challenging RM, that datum was excluded from the mean. The outcome of this exercise was that of 100 results calculated across the laboratories, there was only one 2-s outlier, and that upon inspection of discretionary procedures had an obvious explanation. Intra-laboratory repeatability matched relatively well with inter-laboratory reproducibility, and the value assignments resulting from the exercise were precise, technically sound, and consistent with a consensus mean of globally equitable data.

GC-C-IRMS Research at UCLA

Don Catlin, M.D.
University of California, Los Angeles

Our group became interested in carbon isotope ratio methodologies when Daniel Fraisse, Ph.D., visited UCLA in 1986. Fraisse described that there is a measurable difference in ^{13}C content between natural vanillin, which is expensive, and synthetic vanillin, which is inexpensive and often sold in place of natural vanillin, and that his institution in France had a GC-C-IRMS facility where such vanillin analyses were done. We wondered if there might also be a measurable difference between pharmaceutical and endogenous urinary **T**. By 1990, Southan et al[1] presented an abstract at a meeting in Barcelona that confirmed this possibility. When Fraisse's collaborators Becchi and Aguilera et al[2] reported that in urine collected before and after **T** administration, the $\delta^{13}C$ ("delta values") of urinary **T** and metabolites were significantly lower after **T** administration and that the delta values of metabolic precursors of **T** were unchanged, we began to collaborate with them.

Initially Aguilera joined our group but continued to work in France because we did not have an isotope ratio mass spectrometer (IRMS). It took three more years and several papers[2,3,4] to convince sport funding agencies in the U.S. that the method was sound and could ultimately resolve some of the difficulties with interpretation of T/E ratios that exceed 6. In due course we have verified and validated the diol method in our laboratory, and reported delta values in 73 healthy control subjects.[5,7] We believe it is possible to differentiate between urine samples from athletes with the naturally elevated T/E syndrome and athletes who have used **T**.[7] Although the latter manuscript cannot prove the concept, because we do not yet have good ways to define what the syndrome is, it does suggest that eventually this objective might be obtained.

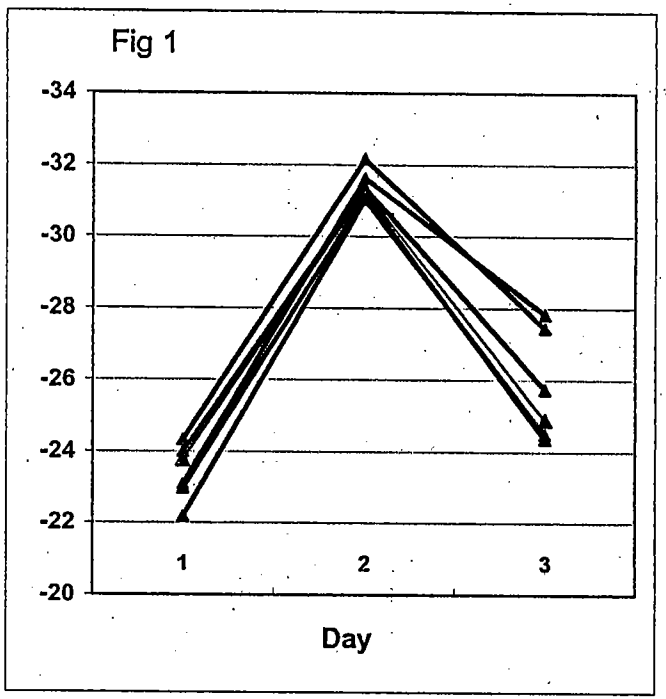
Later we extended the application of the method to **A** and **E**[6] and to epi-T.[8] For T/E cases and for general screening we greatly prefer the Androsterone/**E** assay, even though it does not include an endogenous reference standard, because it is substantially shorter and simpler than the diol assay. Further the low concentrations of diols in some male

http://www.nist.gov/public_affairs/standards.htm. Of particular interest may be **Laboratory Accreditation Activities in the U.S.** (http://ts.nist.gov/ts/htdocs/210/ ncsci/primer1.htm), the **National Voluntary Laboratory Accreditation Program: Procedures and General Requirements** (http://ts.nist.gov/ts/htdocs/210/214/docs/final-hb150-2001.pdf), and the **International Program of Legal Metrology** (http://ts.nist.gov/ts/htdocs/210/tsag/oiml.htm). Note that U.S. accreditation standards have been harmonized with the International Organization of Standards (ISO)/IEC 17025 (Requirements for the Competence of Calibration and Testing Laboratories).

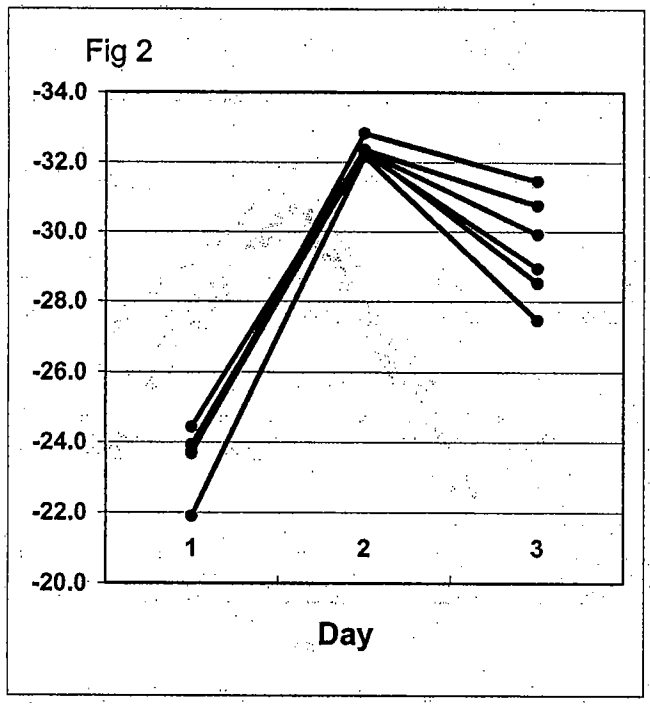
Traceability. The **International Vocabulary of Basic and General Terms in Metrology (ISO VIM, 2nd ed., 1993, entry 6.10)** states that traceability is a *"property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons all having stated uncertainties."* The National Institute of Standards and Technology (NIST) position on this subject is found at http://www.nist.gov/traceability. For $\delta^{13}C$ measurements, the accepted reference of the traceability chain is VPDB, which may be realized only through comparisons that include the carbonate NBS 19 (NIST/ International Atomic Energy Agency [IAEA] Reference Material 8544). Other comparisons in the chain typically involve a non-carbonate Reference Material (RM) standardized against NBS 19, as well as a laboratory material standardized against the RM.

Uncertainty. The **International Vocabulary of Basic and General Terms in Metrology (ISO VIM, 2nd ed., 1993, entry 3.9)** states that uncertainty (of measurement) is a *"parameter, associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand."* Furthermore, uncertainty components are placed in one of two categories, A and B, based on their method of evaluation. Type A standard uncertainty is calculated from a set of repeated observations and is the square root of s^2, the statistically estimated variance. Type B standard uncertainty is obtained from an assumed distribution based on scientific judgment of all possible sources of measurement variability. This is usually based on a pool of comparatively reliable information. The essentials of expressing measurement uncertainty (basic definitions, evaluating uncertainty components, combining uncertainty components, expanded uncertainty, and examples of uncertainty statements may be found in **Guide to the Expression of Uncertainty in Measurement, ISO/ International Bureau of Weights & Measures (BIPM) (1993), Quantifying Uncertainty in Analytical Measurement, Eurachem/ Co-Operation on International Traceability in Analytical**

Figure 1 shows that on day 1, the baseline day, the delta values for the acetate derivatives of urinary A of the six subjects ranged between -22 and -24 $^{0}/_{00}$. On day 2 all delta values peaked between -31 and -32 $^{0}/_{00}$ (close to the maximal theoretical value) for the administered AED. None of the values returned completely to baseline on day 3, but all were becoming less negative indicating that the exogenous substance was being excreted, but still present in some amount.



The pattern for E (Fig 2) is similar except that the values recover considerably slower compared to A. These findings indicate substantial differences between A and E in the clearance of exogenous AED, and offer the possibility that such studies represent an interesting new probe of metabolism of endogenous steroids.



Standardization of Isotope Ratio Measurements for Doping Control

Michael Verkouteren, Ph.D.
NIST

INTRODUCTION

Pharmaceutical steroids are known to differ in isotopic composition from those produced naturally in the body. By measuring the $^{13}C/^{12}C$ ratio of specific steroids detected in urine, the technique of Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) can distinguish between exogenous and natural steroids. As a result, the technique has been designated a definitive test of steroid abuse by the World Anti-Doping Association (WADA) and the USADA, and accepted as evidence in adjudication cases by the IOC. While individual laboratories have successfully applied GC-C-IRMS for isotopic measurements of steroids, little is actually known regarding the reproducibility of these measurements across laboratories since standard practices and reliable reference materials do not exist on which to base an assessment. The IOC and USADA have articulated the need to establish a harmonized international network of laboratories, with each laboratory proven to be equivalent in analytical performance. Measurements from such a network would provide sound and globally equitable data needed to support and strengthen decisions made by IOC officials and foster confidence in those decisions by the athlete and world communities.

This extended abstract will outline briefly some of the metrology issues that should be addressed in order to establish equivalence of analytical results across a laboratory network, discuss the lessons learned from prior exercises of isotopic measurement harmonization, and offer some recommendations specific to this GC-C-IRMS application.

METROLOGY ISSUES: TERMS OF REFERENCE

Defined are selected terms of reference that are central to international chemical assay harmonization. Further development of these terms is beyond the scope of this abstract, but their consideration and integration into the analytical results used for doping control is vital for establishing a harmonized network of laboratories.

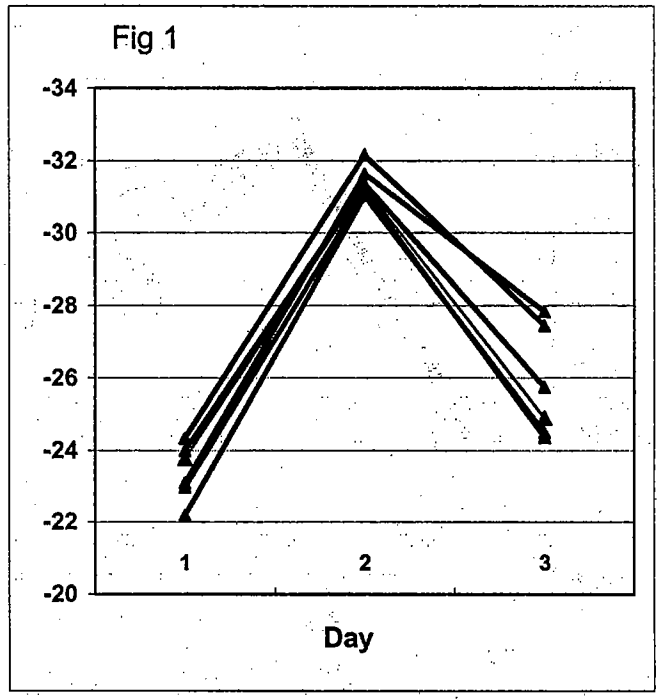
Standards. This term has several meanings in metrology. For detailed information on *measurement standards*, *documentary standards*, and *accreditation standards*, please visit

http://www.nist.gov/public_affairs/standards.htm. Of particular interest may be **Laboratory Accreditation Activities in the U.S.** (http://ts.nist.gov/ts/htdocs/210/ ncsci/primer1.htm), the **National Voluntary Laboratory Accreditation Program: Procedures and General Requirements** (http://ts.nist.gov/ts/htdocs/210/214/docs/final-hb150-2001.pdf), and the **International Program of Legal Metrology** (http://ts.nist.gov/ts/htdocs/210/tsag/oiml.htm). Note that U.S. accreditation standards have been harmonized with the International Organization of Standards (ISO)/IEC 17025 (Requirements for the Competence of Calibration and Testing Laboratories).

Traceability. The **International Vocabulary of Basic and General Terms in Metrology (ISO VIM, 2nd ed., 1993, entry 6.10)** states that traceability is a *"property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons all having stated uncertainties."* The National Institute of Standards and Technology (NIST) position on this subject is found at http://www.nist.gov/traceability. For $\delta^{13}C$ measurements, the accepted reference of the traceability chain is VPDB, which may be realized only through comparisons that include the carbonate NBS 19 (NIST/ International Atomic Energy Agency [IAEA] Reference Material 8544). Other comparisons in the chain typically involve a non-carbonate Reference Material (RM) standardized against NBS 19, as well as a laboratory material standardized against the RM.

Uncertainty. The **International Vocabulary of Basic and General Terms in Metrology (ISO VIM, 2nd ed., 1993, entry 3.9)** states that uncertainty (of measurement) is a *"parameter, associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand."* Furthermore, uncertainty components are placed in one of two categories, A and B, based on their method of evaluation. Type A standard uncertainty is calculated from a set of repeated observations and is the square root of s^2, the statistically estimated variance. Type B standard uncertainty is obtained from an assumed distribution based on scientific judgment of all possible sources of measurement variability. This is usually based on a pool of comparatively reliable information. The essentials of expressing measurement uncertainty (basic definitions, evaluating uncertainty components, combining uncertainty components, expanded uncertainty, and examples of uncertainty statements may be found in **Guide to the Expression of Uncertainty in Measurement, ISO/ International Bureau of Weights & Measures (BIPM) (1993), Quantifying Uncertainty in Analytical Measurement, Eurachem/ Co-Operation on International Traceability in Analytical**

Figure 1 shows that on day 1, the baseline day, the delta values for the acetate derivatives of urinary A of the six subjects ranged between -22 and -24 $^0/_{00}$. On day 2 all delta values peaked between -31 and -32 $^0/_{00}$ (close to the maximal theoretical value) for the administered AED. None of the values returned completely to baseline on day 3, but all were becoming less negative indicating that the exogenous substance was being excreted, but still present in some amount.



Session 3:

Uncertainty in GC-C-IRMS Measurement as Applied to Doping Control

Chair:

Dwight Matthews

REFERENCES

1. Southan G, Mallet J, Jumeau S, Craig N, Poojara D, Mitchell M et al. Unknown. Programme and Abstracts of the Second International Symposium on Applied Mass Spectrometry in Health Sciences, Barcelona, 1990, Abstract, 1990.

2. Becchi M, Aguilera R, Farizon Y, Flament MM, Casabianca H, James P. Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of T in sport. Rapid Commun Mass Spectrom 1994; 8(4):304-308.

3. Shackleton CH, Phillips A, Chang T, Li Y. Confirming T administration by isotope ratio mass spectrometric analysis of urinary androstanediols. Steroids 1997; 62:379-387.

4. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B, Pope HG. Improved method of detection of T abuse by gas chromatography/ combustion/isotope ratio mass spectrometry analysis of urinary steroids. J Mass Spectrometry 1996;31:169-176.

5. Aguilera R, Catlin DH, Becchi M, Phillips A, Wang C, Swerdloff RS, Pope HG, Hatton CK. Screening urine for exogenous T by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols. J Chromatog B 1999;727:95-105.

6. Aguilera R, Chapman TE, Catlin DH. A rapid screening assay for measuring urinary A and E $\delta^{13}(^{0}/_{00})$ values by gas chromatography/combustion /isotope ratio mass spectrometry. Rapid Comm Mass Spec 2000;14:2294-2299.

7. Aguilera R, Chapman TE, Catlin DH. Performance characteristics of a carbon isotope ratio method for detecting doping with T based on urine diols: controls and athletes with elevated T/epi-T ratios. Clin Chem 2001;47:292-300.

8. Aguilera R, Hatton CK, Catlin DH. Detection of doping with epi-T by isotope ratio mass spectrometry. Clin Chem 2002;48:629-636.

Question & Answer Discussion

AUCHUS
Does this Asian pattern run in families? Can you score it as a quantitative trait and then do linkage analysis to find the gene for 17α-HSDs?

CATLIN
I wish I could answer that. We have one person, who happens to be Caucasian, who falls into this pattern. He sends us samples from his sons and daughters. It is not very clear.

AUCHUS
The pattern where you see the 5α Adiol but not the 5β Adiol, couldn't that be explained by someone taking a combination of DHT and T, and shouldn't that be reported as positive?

CATLIN
It could be explained that way and other ways. It is the "shouldn't it be proven as positive" that is the problem. Because as soon as your report it positive you have to prove it. Not only do you have to prove it, you better well have a lot of clinical studies that demonstrate it or you will lose in the CAS.

AUCHUS
Has anyone taken the Chinese samples from the Asian games where the Mitsubishi assays were used for the 5α/5β and gone back and run them with carbon isotope ratio?

BOWERS
We will hear about that in a few minutes from Dr. Ueki.

MATTHEWS
Back to the Asian syndrome and the 15% of Caucasians. You know you are getting the T in because you see the results in the metabolites and in the urine. But simply put, where does the T go? What pathway does it follow through, if it is not appearing in the urine?

- It may be necessary for a committee to establish one method for GC-C-IRMS analysis
- There may be benefit in having multiple methods and multiple ERCs to make it more difficult to avoid detection.
- $\delta^{13}C$ values obtained from derivatized steroids should be corrected for contributions of the derivatizing agent, possibly by reference compound of known isotopic composition

- Reference ranges for target compounds for individual laboratories for populations with different diets is a concern
  - Athlete may change location or diet according to whether they are training, competing, or resting
  - Laboratory does not know the dietary "history" or geographic origin of the athlete competing

REFERENCES

1. Flenker U, Schänzer W. Expression of differences in isotopic composition. In Recent Advances in Doping Analysis (11), W Schänzer, H Geyer, A Gotzmann, U. Mareck, eds., Sport and Buch Strauß, Köln, 2003. pp 179-182.

Session 3:

Uncertainty in GC-C-IRMS Measurement as Applied to Doping Control

Chair:

Dwight Matthews

REFERENCES

1. Southan G, Mallet J, Jumeau S, Craig N, Poojara D, Mitchell M et al. Unknown. Programme and Abstracts of the Second International Symposium on Applied Mass Spectrometry in Health Sciences, Barcelona, 1990, Abstract, 1990.

2. Becchi M, Aguilera R, Farizon Y, Flament MM, Casabianca H, James P. Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of T in sport. Rapid Commun Mass Spectrom 1994; 8(4):304-308.

3. Shackleton CH, Phillips A, Chang T, Li Y. Confirming T administration by isotope ratio mass spectrometric analysis of urinary androstanediols. Steroids 1997; 62:379-387.

4. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B, Pope HG. Improved method of detection of T abuse by gas chromatography/ combustion/isotope ratio mass spectrometry analysis of urinary steroids. J Mass Spectrometry 1996;31:169-176.

5. Aguilera R, Catlin DH, Becchi M, Phillips A, Wang C, Swerdloff RS, Pope HG, Hatton CK. Screening urine for exogenous T by isotope ratio mass spectrometric analysis of one pregnanediol and two androstanediols. J Chromatog B 1999;727:95-105.

6. Aguilera R, Chapman TE, Catlin DH. A rapid screening assay for measuring urinary A and E $\delta^{13}(^0/_{00})$ values by gas chromatography/combustion /isotope ratio mass spectrometry. Rapid Comm Mass Spec 2000;14:2294-2299.

7. Aguilera R, Chapman TE, Catlin DH. Performance characteristics of a carbon isotope ratio method for detecting doping with T based on urine diols: controls and athletes with elevated T/epi-T ratios. Clin Chem 2001;47:292-300.

The application of GC-C-IRMS has proceeded in different directions in the eleven (11) WADA/IOC-accredited laboratories that are using the technique. The following elements contribute to observed differences between the laboratories:

- Sample clean-up procedures
- Derivatized (e.g., diacetates) versus underivatized target compounds
- Instrumentation [GV Intruments (formerly MicroMass), PDZ Europa, ThermoFinnigan] and instrumental conditions
- Several target compounds for **T** and its precursors
  - **A** and **E** (average or individual values)
  - 5α- and 5β-Androstan-3α,17β-diol
  - **T**
- ERC – originally proposed by Shackleton as pregnanediol and pregnanetriol. Others include 11-ketoetiocholanolone and 11-hydroxyetiocholanolone.
- There have been several different approaches to detecting an administration of **T** or its precursors
  - $\delta^{13}C$ reference range for each compound
  - Difference between the $\delta^{13}C$ values for the target compound and an ERC
  - Ratio of the $\delta^{13}C$ values for the index compound and an ERC
  - Combinations of the above
- Use of internal standard compounds
  - 5α-androstan-3β-ol
  - Octacosane
  - Androstanol
  - 17α-methyltestosterone
  - 17α-methylandrostan-3α,17β-diol
- Selected other target compounds for doping agents other than **T** and its precursors
  - 5α- and 5β-Androstan-3α,17α-diol (for epi-T)
  - Androst-5-ene-3β,17β-diol (for DHEA)
  - NorAndrosterone for nortestosterone and its precursors in very rare cases where the concentration is sufficiently high to allow application of the GC-C-IRMS technique

The following points were made during the discussion:

- The reporting of differences of delta values as opposed to the ratio of delta values is strongly recommended (in addition, see reference [1])
- Early studies used acetate derivatives to improve the chromatography, but the situation has improved and good chromatographic peak shapes can be obtained with underivatized steroids

CATLIN
We think we are experts in urine, and I suppose we are experts in urine. When you take **T**, 99% does not show up as **T** in urine. We are looking at a very tiny fraction. Most of our assays are just looking at the glucuronide (**G**) fraction. If you read through the literature, people who have been given infusions of **T**, way back 40 years ago, give you a good idea of pathways.

It is all changing now; you really can't say where each molecule goes of those 99 that are given intravenously. You know the major pathway. But we are only looking at the 1% that gets into the urine and gets glucuronidated and to some extent sulfated.

MATTHEWS
It seems to me if you could, for example, knock out the ability to form the **G** then you could get around the T/E ratio test. You could open up a big hole if you naturally didn't make the **G**.

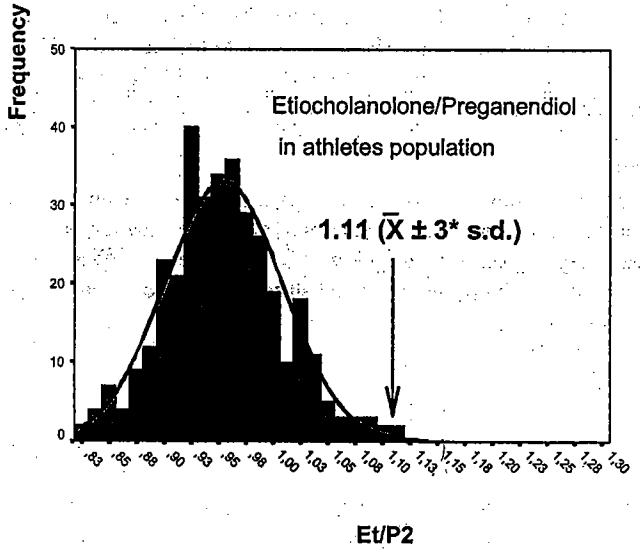
CATLIN
We could have a whole conference on how to beat the test. It may be possible for a serious scientist to develop a way to foil the GC-C-IRMS test. We are trying to do it ourselves because we would like to know before the bad guys do.

BOWERS
Some years ago we did LC-MS-MS to **G**s and sulfates of **T** and epi-**T**. They are both present in Asian populations. Although they are some what lower, they are not very, very low. Clearly there is a difference in metabolism but not glucuronidation and sulfation; it is somewhere else in the pathway.

GC-C-IRMS for endogenous steroids "Challenging the System"

Jordi Segura
Institut Municipal Investigacio Medica

Natural androgens excreted in human urine, may have a higher content of ^{13}C compared with the synthetic ones (normally derived from soy phytosteroids) and consequently their $^{13}C/^{12}C$ ratio may be different. The detection of a modified $^{13}C/^{12}C$ ratio in urinary **T** or its metabolites could be a direct evidence of the exogenous intake of synthetic **T**, precursors or metabolites. Differences in $^{13}C/^{12}C$ ratio can be determined by carbon isotope ratio mass spectrometry (IRMS). For complex mixtures, this technique can be coupled with a gas chromatograph via a combustion interface (GC-C). Separated organic analytes are combusted to CO_2 and the analysis of the $^{13}CO_2$ and $^{12}CO_2$ molecules allows the calculation of carbon isotope ratios for each compound. These minute differences in carbon isotope ratios are expressed as a per mil deviation (δ ^{13}C ‰) compared to a designated isotopic standard



In this study, gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) analysis for the measurement of the δ ^{13}C

Summary of Discussion:

Basics and Application of GC-C-IRMS in Doping Control

The application of GC-C-IRMS has proceeded in different directions in the eleven (11) WADA/IOC-accredited laboratories that are using the technique. The following elements contribute to observed differences between the laboratories:

- Sample clean-up procedures
- Derivatized (e.g., diacetates) versus underivatized target compounds
- Instrumentation [GV Intruments (formerly MicroMass), PDZ Europa, ThermoFinnigan] and instrumental conditions
- Several target compounds for **T** and its precursors
  - **A** and **E** (average or individual values)
  - 5α- and 5β-Androstan-3α,17β-diol
  - **T**
- ERC – originally proposed by Shackleton as pregnanediol and pregnanetriol. Others include 11-ketoetiocholanolone and 11-hydroxyetiocholanolone.
- There have been several different approaches to detecting an administration of **T** or its precursors
  - $\delta^{13}C$ reference range for each compound
  - Difference between the $\delta^{13}C$ values for the target compound and an ERC
  - Ratio of the $\delta^{13}C$ values for the index compound and an ERC
  - Combinations of the above
- Use of internal standard compounds
  - 5α-androstan-3β-ol
  - Octacosane
  - Androstanol
  - 17α-methyltestosterone
  - 17α-methylandrostan-3α,17β-diol
- Selected other target compounds for doping agents other than **T** and its precursors
  - 5α- and 5β-Androstan-3α,17α-diol (for epi-T)
  - Androst-5-ene-3β,17β-diol (for DHEA)
  - NorAndrosterone for nortestosterone and its precursors in very rare cases where the concentration is sufficiently high to allow application of the GC-C-IRMS technique

The following points were made during the discussion:

- The reporting of differences of delta values as opposed to the ratio of delta values is strongly recommended (in addition, see reference [1])
- Early studies used acetate derivatives to improve the chromatography, but the situation has improved and good chromatographic peak shapes can be obtained with underivatized steroids

CATLIN

We think we are experts in urine, and I suppose we are experts in urine. When you take **T**, 99% does not show up as **T** in urine. We are looking at a very tiny fraction. Most of our assays are just looking at the glucuronide (**G**) fraction. If you read through the literature, people who have been given infusions of **T**, way back 40 years ago, give you a good idea of pathways.

It is all changing now; you really can't say where each molecule goes of those 99 that are given intravenously. You know the major pathway. But we are only looking at the 1% that gets into the urine and gets glucuronidated and to some extent sulfated.

MATTHEWS

It seems to me if you could, for example, knock out the ability to form the **G** then you could get around the T/E ratio test. You could open up a big hole if you naturally didn't make the **G**.

CATLIN

We could have a whole conference on how to beat the test. It may be possible for a serious scientist to develop a way to foil the GC-C-IRMS test. We are trying to do it ourselves because we would like to know before the bad guys do.

BOWERS

Some years ago we did LC-MS-MS to **G**s and sulfates of **T** and epi-**T**. They are both present in Asian populations. Although they are some what lower, they are not very, very low. Clearly there is a difference in metabolism but not glucuronidation and sulfation, it is somewhere else in the pathway.

In conclusion, the GC-C-IRMS usefulness applied to natural steroid control in breeding animals has been demonstrated. Some improvements are still necessary to make the technique more efficient (instrumental robustness, sensitivity, validation, criteria of positivity...) and finally applicable to matrices more challenging in terms of residue levels.

Question & Answer Discussion

BRENNA
PTV injection, what does stand for?

LI BIZEC
Large volume injector (Programmed Temperature Volatilization)

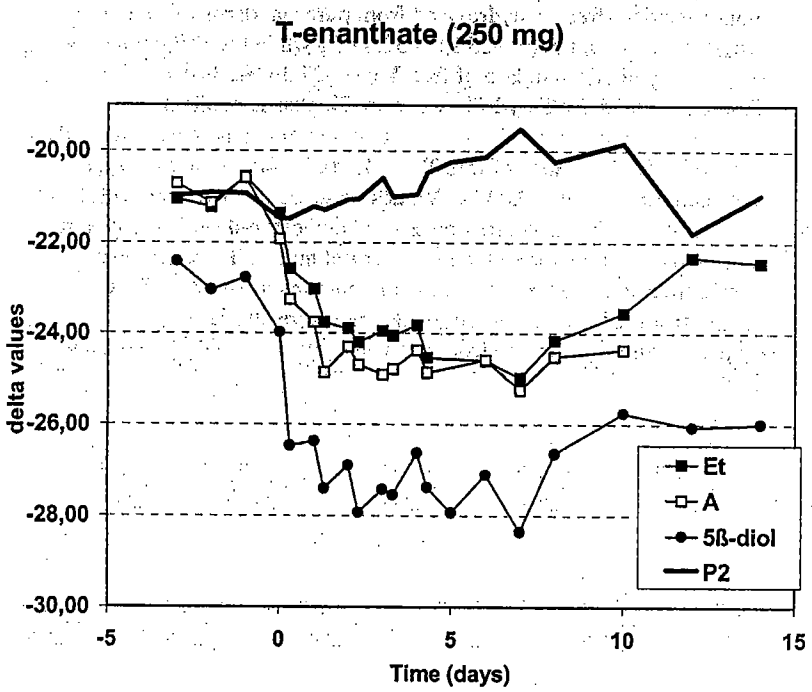
HALE
You were using this with cattle. There are a lot of differences in cattle, bulls, females, and most importantly the neutered cattle. Did you make any different change in account for that? Second question, you showed some very interesting slides on **T**, but you also said you used estrAdiol. But I did not see any extra slides on estrAdiol, and how that had an impact on the cattle and steroidogenesis.

LE BIZEC
What we are doing in Austria with the project ISOSTER, It is not protected, but all of the results must be agreed upon before verification. Can you explain the first question?

HALE
In the US, we take a male cow and take off the testes. Was that type of animal being studied or were you studying animals with in-tact gonads. Castrated versus not castrated animals?

LE BIZEC
In general what is practiced in farms is that I always compare to the opposite gender. So I used castrated males and females. I have been presented with web data that explains that there is no real difference. There is not a significant difference between castrated males and females.

‰ value of **T** obtained from esterified forms of pharmaceutical preparations and pure standards obtained world-wide was investigated after applying different hydrolytic procedures. Mean $\delta\ ^{13}C$ ‰ values of non-esterified (free) **T** hydrolysed from pure standards of several **T** esters were in the range: –25.91/ - 32.82 ‰ while the value obtained for a (semi)-synthetic standard of free **T** was -27.36 ‰. The $\delta\ ^{13}C$ ‰ results obtained for **T** hydrolysed from the pharmaceuticals investigated containing **T** esters were quite homogeneous (mean and S.D. of $\delta\ ^{13}C$ ‰ values of free **T**: 27.43±0.76 ‰), being the range between -26.18 and -30.04 ‰. Values described above were clearly different from those reported by several authors and studied by us in athlete populations for endogenous natural human **T** and its main metabolites excreted into the urine in non-consumers of **T** ($\delta\ ^{13}C$ ‰, range: from -21.3 to -24.4 ‰), while they were similar to those of urinary **T** and metabolites from individuals treated with **T** esters and **T** precursors.



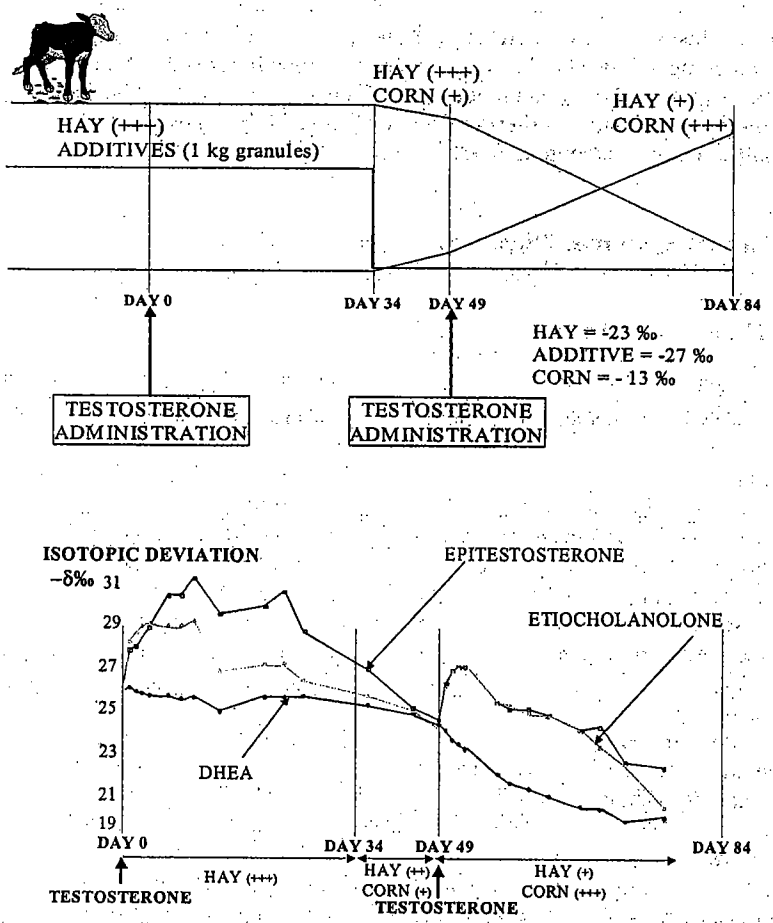


Figure 2: Evaluation of dietary changes over the CIR of ERC and metabolites (above: protocol of the experimentation; underneath: isotopic deviation versus time).

In conclusion, the GC-C-IRMS usefulness applied to natural steroid control in breeding animals has been demonstrated. Some improvements are still necessary to make the technique more efficient (instrumental robustness, sensitivity, validation, criteria of positivity…) and finally applicable to matrices more challenging in terms of residue levels.

Question & Answer Discussion

BRENNA
PTV injection, what does stand for?

LI BIZEC
Large volume injector (Programmed Temperature Volatilization)

HALE
You were using this with cattle. There are a lot of differences in cattle, bulls, females, and most importantly the neutered cattle. Did you make any different change in account for that? Second question, you showed some very interesting slides on T, but you also said you used estrAdiol. But I did not see any extra slides on estrAdiol, and how that had an impact on the cattle and steroidogenesis.

LE BIZEC
What we are doing in Austria with the project ISOSTER, It is not protected, but all of the results must be agreed upon before verification. Can you explain the first question?

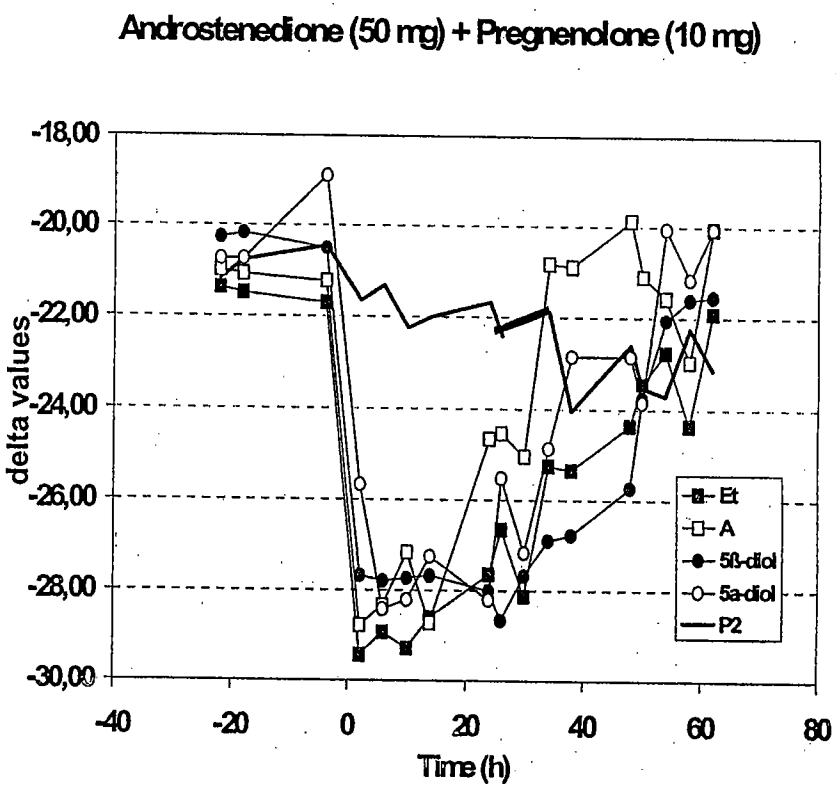
HALE
In the US, we take a male cow and take off the testes. Was that type of animal being studied or were you studying animals with in-tact gonads. Castrated versus not castrated animals?

LE BIZEC
In general what is practiced in farms is that I always compare to the opposite gender. So I used castrated males and females. I have been presented with web data that explains that there is no real difference. There is not a significant difference between castrated males and females.

‰ value of T obtained from esterified forms of pharmaceutical preparations and pure standards obtained world-wide was investigated after applying different hydrolytic procedures. Mean $\delta^{13}C$ ‰ values of non-esterified (free) T hydrolysed from pure standards of several T esters were in the range: -25.91/ -32.82 ‰ while the value obtained for a (semi)-synthetic standard of free T was -27.36 ‰. The $\delta^{13}C$ ‰ results obtained for T hydrolysed from the pharmaceuticals investigated containing T esters were quite homogeneous (mean and S.D. of $\delta^{13}C$ ‰ values of free T: 27.43±0.76 ‰), being the range between -26.18 and -30.04 ‰. Values described above were clearly different from those reported by several authors and studied by us in athlete populations for endogenous natural human T and its main metabolites excreted into the urine in non-consumers of T ($\delta^{13}C$ ‰ range: from -21.3 to -24.4 ‰), while they were similar to those of urinary T and metabolites from individuals treated with T esters and T precursors.

improvable. The first idea was to study the classical silylating mixture MSTFA/TMIS/ dithiothreitol (DTT). Nevertheless, poor $^{13}C/^{12}C$ repeatability was observed and attributed to the catalyst which was presumed to compete with MSTFA during the silylation. The replacement of TMIS with NH_4I led us to determine a new reagent: MSTFA/NH_4I/DTT/CH_3CN (1000:5:0.25:100, v/w/w/v). Inter-day reproducibility of derivatized target steroids on standard was judged acceptable (< ± 0.5‰); peak width was improved and IRMS signal intensities (m/z 44) was in the 5.10^{-9} A order for 20 ng injected. Combustion furnace life is not significantly affected (± 6 months) especially when the effluent gas is vented before entering the combustion furnace. Extracts are injected into the gas chromatograph through a split/splitless injector, and separated onto a capillary column (Macherey-Nagel, δ-3, 30 m 0.25 mm x 0.25 µm).

In bovine urine, two ERCs, four T metabolites, and two estrAdiol metabolites are excreted in sufficiently high amount to be measurable in most cases by GC-C-IRMS. We demonstrated the ability of the method to differentiate ERC and at least two T metabolites on the basis of their $^{13}C/^{12}C$ over a period of 2-3 weeks in bovine urine (whatever breed and age) after IM injection of T esters (250 mg single dose). At this level, we decided to evaluate the consequences of dietary modification on the isotopic deviations of ERC and metabolites. For that (Figure 2), a cow (Normande, 15 years) received a diet based on hay and granules (1 kg daily) during several weeks (period until Day 34), corn replaced granules (Day 34), then corn proportion was slightly augmented (Day 34 to Day 49) and finally considerably increased into the feeding (Day 40 to Day 84). T enanthate (250 mg) was IM injected at Day 0 and Day 49. EA-IRMS of hay, granules and corn gave –23‰, -27‰ and –13‰, respectively. Results clearly showed on the first period (until Day 34) that the ERC remained constant (-25‰). The ^{13}C composition of the two metabolites obviously decreased after T administration and remained significantly different from the ERC over several weeks. The slight diet modification (Day 34 to Day 49, maize introduction) generated a perceptible ^{13}C enrichment of DHEA (± 1‰). Then the massive introduction of corn into the diet noticeably enriched the ^{13}C composition of DHEA. The T injection (Day 49) produced a clear $^{13}C/^{12}C$ modification of both metabolites (maximum (± 4‰) in-between metabolites and precursors). The misuse detectability remained the same in the two phases of the experiment.



A comprehensive analytical method using GC-C-IRMS allowing the detection of these compounds overcoming the inter-individual variability in steroid profile excretion or metabolism in routine conditions for sports drug testing has been developed. The method proved to be able to detect controlled administrations by different routes of T, dihydrotestosterone (DHT), AED, and dehydroepiAndrosterone (DHEA) in healthy volunteers for longer periods than using official criteria, when existing. The obtained data were compared with reference values obtained by healthy non-steroid abusers. Even with the concomitant administration of pregnenolone, masking the normal isotopic content of the natural marker preganendiol, the method is able to clearly demonstrate the ingestion of T precursors.

In the absence of blood markers of T abuse (testosterone esters, G of testosterone metabolites), GC-C-IRMS appears as one of the major tools to detect doping by T or T precursors.

Question & Answer Discussion

MATTHEWS
From the perspective of methodologies such as GC/MS where we are used to thinking of the wider linear range of quantization than we are with GC-C-IRMS, I was impressed that you had selected a wide range for your isotope ratio measurements. My experience with GC-C-IRMS is that you have a much more limited range in which you can work in for precise ^{13}C enrichment measurements. The $\delta^{13}C$ values that you put up, as a function of concentration, actually were quite good. You had a good range of quantification. What do you think?

SEGURA
Yes, we were not so upset by the range; we obtained the range that is wider than may be expected. We were really disappointed by the low extreme of the range, and we expected we could reach the stability of delta values with lower concentration. We injected less amounts than we in fact needed.

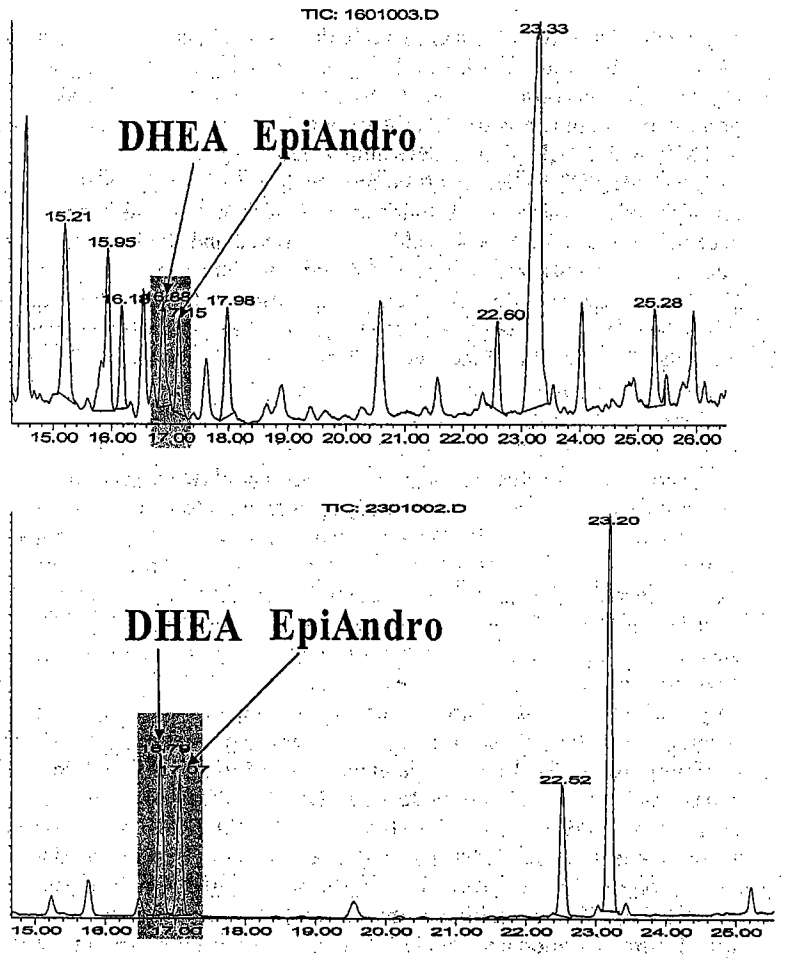
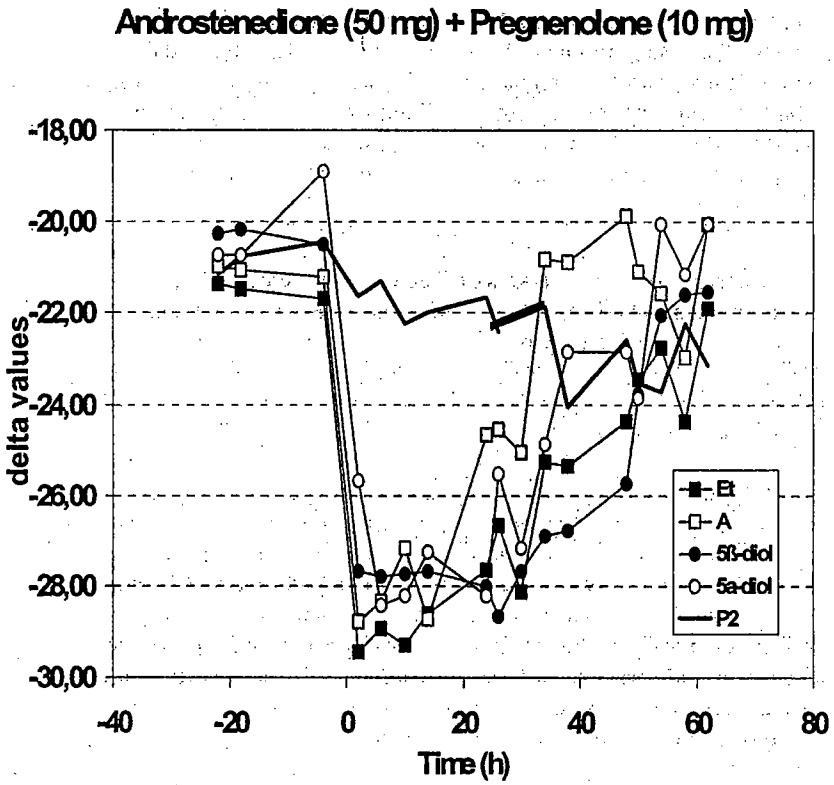


Figure 1: Purification efficiency of a semi-preparative HPLC (aminopropyle stationary phase) prior to any GC-C-IRMS measurement.

On one hand, acetylation derivatization is widely used for steroid analysis by GC-C-IRMS because it leads to good chromatographic behaviour (compared to non-derivatized steroid) without affecting the carbon isotope ratio of the target analyte (two carbon atoms per derivatized site). The analysis of hundreds of samples led us to occasionally observe incomplete derivatization (mixture of mono- and di-acetylated steroids), and the chromatographic behaviour of the derivatized steroids was judged

improvable. The first idea was to study the classical silylating mixture MSTFA/TMIS/ dithiothreitol (DTT). Nevertheless, poor $^{13}C/^{12}C$ repeatability was observed and attributed to the catalyst which was presumed to compete with MSTFA during the silylation. The replacement of TMIS with NH_4I led us to determine a new reagent: $MSTFA/NH_4I/DTT/CH_3CN$ (1000:5:0.25:100, v/w/w/v). Inter-day reproducibility of derivatized target steroids on standard was judged acceptable (< ± 0.5‰); peak width was improved and IRMS signal intensities (m/z 44) was in the 5.10^{-9} A order for 20 ng injected. Combustion furnace life is not significantly affected (± 6 months) especially when the effluent gas is vented before entering the combustion furnace. Extracts are injected into the gas chromatograph through a split/splitless injector, and separated onto a capillary column (Macherey-Nagel, δ-3, 30 m 0.25 mm x 0.25 µm).

In bovine urine, two ERCs, four T metabolites, and two estrAdiol metabolites are excreted in sufficiently high amount to be measurable in most cases by GC-C-IRMS. We demonstrated the ability of the method to differentiate ERC and at least two T metabolites on the basis of their $^{13}C/^{12}C$ over a period of 2-3 weeks in bovine urine (whatever breed and age) after IM injection of T esters (250 mg single dose). At this level, we decided to evaluate the consequences of dietary modification on the isotopic deviations of ERC and metabolites. For that (Figure 2), a cow (Normande, 15 years) received a diet based on hay and granules (1 kg daily) during several weeks (period until Day 34), corn replaced granules (Day 34), then corn proportion was slightly augmented (Day 34 to Day 49) and finally considerably increased into the feeding (Day 40 to Day 84). T enanthate (250 mg) was IM injected at Day 0 and Day 49. EA-IRMS of hay, granules and corn gave –23‰, -27‰ and –13‰, respectively. Results clearly showed on the first period (until Day 34) that the ERC remained constant (-25‰). The ^{13}C composition of the two metabolites obviously decreased after T administration and remained significantly different from the ERC over several weeks. The slight diet modification (Day 34 to Day 49, maize introduction) generated a perceptible ^{13}C enrichment of DHEA (± 1‰). Then the massive introduction of corn into the diet noticeably enriched the ^{13}C composition of DHEA. The T injection (Day 49) produced a clear $^{13}C/^{12}C$ modification of both metabolites (maximum (± 4‰) in-between metabolites and precursors). The misuse detectability remained the same in the two phases of the experiment.



GC-C-IRMS to Control the Misuse of Natural Occurring Steroid in Breeding Animals

Bruno Le Bizec, Corinne Buisson, Véronique Ferchaud, Stéphanie Prevost, Tatiana Nicol, Marie-Aude Kerautret, Bruno Veyrand, Fabrice Monteau and François Andre
French Reference Laboratory

The use of anabolic growth promoting preparations for improving the growth and feed conversion rates of food producing animals has been banned within the European Union since 1988[1]. Whereas the use of strictly xenobiotic steroids decreased since the end of the 90's, the illegal application of natural steroid hormones remains a current practice probably because the classical analytical methodologies developed up till now and applied daily in the control laboratories are still not rigorous enough for this purpose. Because current approaches involve the determination of the absolute concentration, in most cases the results cause controversy. Indeed, no real and validated physiological levels are officially recognised. Other attempts based on hair analysis and demonstration of the steroid esters residues are currently used, but this strategy excludes some steroids (progesterone, norandrostenedione) and becomes non applicable when non-esterified natural occurring steroids are administered.

The most promising and definitive approach remains the determination of the isotopic $^{13}C/^{12}C$ composition of steroids, and the examination of any significant variation in-between ERCs and monitored target metabolites. To be unambiguously applicable to the control, the technique requires a lot of precautions from the sample preparation (extraction, purification and derivatization), to the final IRMS measurement (injection, chromatographic separation and $^{13}C/^{12}C$ determination). Biological samples collected from breeding animals (mainly bovine urine) are particularly complex. The analytical strategy developed in our laboratory since several years includes necessarily at least 2 SPE steps based on complementary chemical principles (C_{18}, SiOH), and a final semi-preparative HPLC (NH_2). This step authorizes drastic purification of the extract (figure 1); nevertheless, there is a clear associated fractionation risk. Even whether the fraction collection should be enlarged to avoid any $^{13}C/^{12}C$ discrimination, the introduction of one internal standard in each collected fraction prevents any troubles.

MATTHEWS
We are trying to measure a carbon isotope, where the isotope is 100-fold lower than what we normally think of, which is the m/z 45 beam. So when we get down toward the low end, the m/z 45 beam is 100 times lower than the m/z 44, and it is really, really low. I think we are doing very well with a 100-fold linear range. So from my experience, I think what you show is not surprising at all. I think it is excellent that the method is working as well as you have been able to make it work.

Improved detection of T cascades by monitoring delta-parameters of A, etiocholanolone, 5 α- and 5 β-androstandiols and DHEA

Makoto Ueki, Ph.D.
Mitsubishi Kagaku Bio-Clinical Laboratories

A typical characteristic of steroid profiles of East Asians such as Japanese, Chinese and Korean is extremely low T/E ratio and poor response of their system to administration of **T**. Such difference makes tests for doping with naturally occurring steroids difficult. Ethnic differences in metabolic patterns of precursor steroids have also been observed. Carbon isotope ratio measurement in doping control is particularly useful to differentiate administered synthetic steroids from these with human origin, and doping with **T** precursors can be definitively detected by monitoring delta-parameters of some gateway steroids such as **A**, **E**, 5α- and 5β-androstan-3α,17-β-diols(5aA2, 5bA2), and DHEA.

Pregnenolone is not a fully formed steroid hormone, and its abuse as a source of anabolic steroid is unlikely; however, administration of pregnenolone results in excretion of pregnanediol and –triol; suggesting it can be abused as a masking agent to alter delta- values of ERCs. Our results suggest that normal steroid profiling alone does not constitute scientific evidence of pregnenolone doping, but reduced ^{13}C-content of pregnanediol (P2) or pregnanetriol (P3) relative to that of any of androgenic steroid could be of the sign of its doping. Carbon isotope ratio testing is sufficiently conclusive even if it is a single test. A second carbon isotope ratio test with few days interval may also be useful to follow a suspicious case of pro-hormones abuse. This is useful because a major part of orally administered steroids are eliminated from the body in several days, but intra-individual variation of carbon isotope ratio in human steroids is very small. An acute change of their delta parameters would indicate doping with **T** precursors.

with what you eat and its relative carbon source. It all gets eventually down to the carbon, liver, and the TCA cycle, makes into what we need to make glucose and fat, and there you go.

When you do these kinds of studies, you could do them very simply. You can take breath samples of a whole variety of populations. If you believe you are what you eat, then the same differences should be seen in breath CO_2. There a quite a few older studies that shows the differences between Asians, Europeans, and Americans. You can tell a lot about what the diet of the person is just from the breath CO_2.

GC-C-IRMS to Control the Misuse of Natural Occurring Steroid in Breeding Animals

Bruno Le Bizec, Corinne Buisson, Véronique Ferchaud, Stéphanie Prevost, Tatiana Nicol, Marie-Aude Kerautret, Bruno Veyrand, Fabrice Monteau and François Andre
French Reference Laboratory

The use of anabolic growth promoting preparations for improving the growth and feed conversion rates of food producing animals has been banned within the European Union since 1988[1]. Whereas the use of strictly xenobiotic steroids decreased since the end of the 90's, the illegal application of natural steroid hormones remains a current practice probably because the classical analytical methodologies developed up till now and applied daily in the control laboratories are still not rigorous enough for this purpose. Because current approaches involve the determination of the absolute concentration, in most cases the results cause controversy. Indeed, no real and validated physiological levels are officially recognised. Other attempts based on hair analysis and demonstration of the steroid esters residues are currently used, but this strategy excludes some steroids (progesterone, norandrostenedione) and becomes non applicable when non-esterified natural occurring steroids are administered.

The most promising and definitive approach remains the determination of the isotopic $^{13}C/^{12}C$ composition of steroids, and the examination of any significant variation in-between ERCs and monitored target metabolites. To be unambiguously applicable to the control, the technique requires a lot of precautions from the sample preparation (extraction, purification and derivatization), to the final IRMS measurement (injection, chromatographic separation and $^{13}C/^{12}C$ determination). Biological samples collected from breeding animals (mainly bovine urine) are particularly complex. The analytical strategy developed in our laboratory since several years includes necessarily at least 2 SPE steps based on complementary chemical principles (C_{18}, SiOH), and a final semi-preparative HPLC (NH_2). This step authorizes drastic purification of the extract (figure 1); nevertheless, there is a clear associated fractionation risk. Even whether the fraction collection should be enlarged to avoid any $^{13}C/^{12}C$ discrimination, the introduction of one internal standard in each collected fraction prevents any troubles.

MATTHEWS
We are trying to measure a carbon isotope, where the isotope is 100-fold lower than what we normally think of, which is the m/z 45 beam. So when we get down toward the low end, the m/z 45 beam is 100 times lower than the m/z 44, and it is really, really low. I think we are doing very well with a 100-fold linear range. So from my experience, I think what you show is not surprising at all. I think it is excellent that the method is working as well as you have been able to make it work.

AHRENS
Actually, the diols were measured for the individuals. The problem with the diol measurements were that there were several instances where concentrations of the diols were too low to get to what we would be confident that the diols were accurate measurement of delta values.

UEKI
I have been following my own delta value for the past five years. The difference was within one unit, only. I have started, three months ago, a high soy diet, and even after losing 14 kilograms of body weight, the delta value does not change at all. My explanation is that the carbon source of steroids does not come from soy. A small amount of meat, cholesterol is enough to generate. This is my understanding.

AUCHUS
Were you referring to the ^{13}C of the diols or were you talking about the ^{13}C of the total plant?

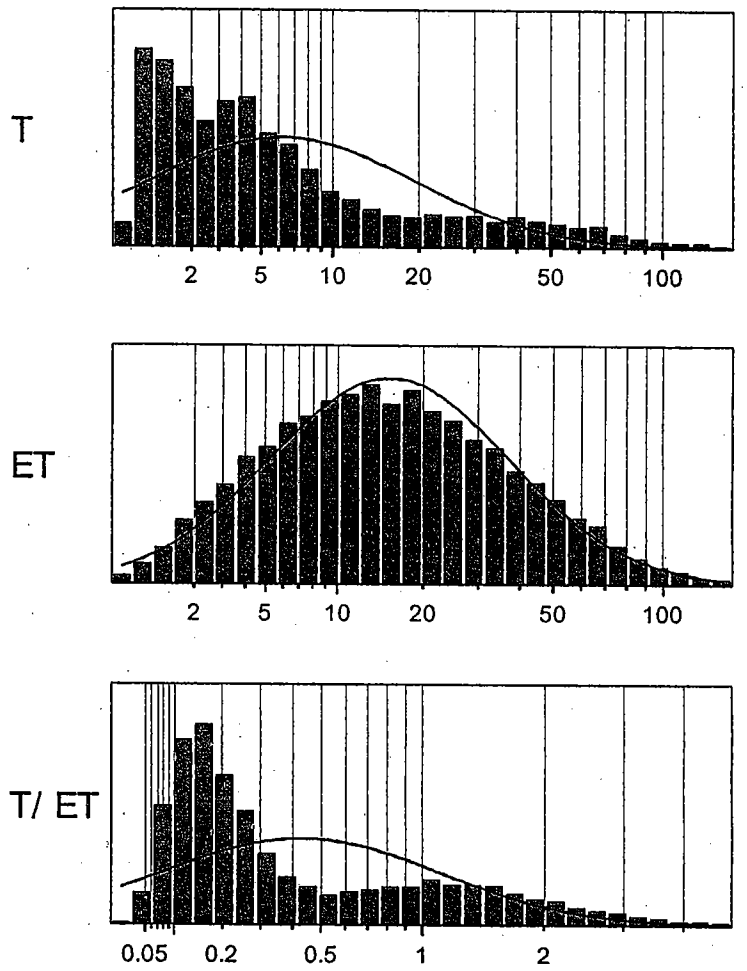
AHRENS
I believe it was the ^{13}C content of the plant, not the specifically of the sterols.

AUCHUS
What is the acetate? The acetate is going into the cholesterol that you are making. So again, the synthetic steroids are reflecting the sterol content unit which is not necessarily what is going into your body and then going into your cholesterol and then your sterol. I think that is the correct explanation, basically.

MATTHEWS
You are right about the acetate. You are what you eat. Same thing for these plants. They are what they fix. They are isotopiclly light or heavy all the way through. So their acetate is isotopically light or heavy all the way through. The acetate may be a little lighter relatively to glucose, for example in these plants, but the overall plant is either isotopiclly light or heavy.

This brings us back to the issue of corn. Because, I would suggest that if an Asian person who is not on a corn diet to begin with shifts his protein source around a little bit going to soy, there would almost be no change in the delta value of his steroids. Because it is probably the corn which is the most important aspect. It has nothing to do with the meat. It just has to do

Figure-1 Distributions of T parameters in international sports (n=12,388: ng/ml)



Figures 2, 3, 4; Closed lines correspond to delta-values after administration of synthetic steroids. Dotted lines show delta values of basal level. Experiments were done with 3 each of females and males. Data from the typical case are presented.

Abbreviations:
Suffices "T", "S" and "G" means steroid fractions of total, sulfate and **G** respectively. Significant differences of delta-parameter of steroids of interest between fractions were not observed

In summary, farm subjects who subsist on an all-soy diet have more negative **A** and **E** $\delta^{13}C$ values compared to a controls. Further studies of diet and populations are planned.

REFERENCES

1. Aguilera R, Chapman TE, Catlin DH. A rapid screening assay for measuring urinary **A** and **E** $\delta^{13}(^{0}/_{00})$ values by gas chromatography/combustion /isotope ratio mass spectrometry. Rapid Comm Mass Spec 2000;14:2294-2299.

Question & Answer Discussion

MATTHEWS
So when the dietician interviewed these people, how much corn did they use? They probably would not be eating that much sweet corn, especially in the winter. So it would be corn meal only, so they should be relatively corn free since they are not eating meat that is coming from a corn fed diet.

AHRENS
This is true. We do have the interviews from the dietician. Our target at that point, when the study was set up; we weren't focusing on corn at all. Our thought was basically soy. Most of the questions that were asked of the study subjects were, "How much soy do you take in, in your daily diet?" Every item that the individual would eat that contained any amount of soy was than further analyzed to determine kilocalories of soy material. I cannot tell you right now how much corn was taken in by either the control or the vegan population.

SEGURA
How do these people in high soy diets respond to an administration of **T**? If they go out of the population range and especially an internal marker would help in the differentiation. Have you planned to do some administration of this personal population?

AHRENS
I would love to be able to do that. I cannot say that there are any plans to undertake that study.

BOWERS
You chose to use the A/E, is there a reason why you choose to do that? Why not in addition to the diols, if you had the option to do that?

AHRENS
Actually, the diols were measured for the individuals. The problem with the diol measurements were that there were several instances where concentrations of the diols were too low to get to what we would be confident that the diols were accurate measurement of delta values.

UEKI
I have been following my own delta value for the past five years. The difference was within one unit, only. I have started, three months ago, a high soy diet, and even after losing 14 kilograms of body weight, the delta value does not change at all. My explanation is that the carbon source of steroids does not come from soy. A small amount of meat, cholesterol is enough to generate. This is my understanding.

AUCHUS
Were you referring to the ^{13}C of the diols or were you talking about the ^{13}C of the total plant?

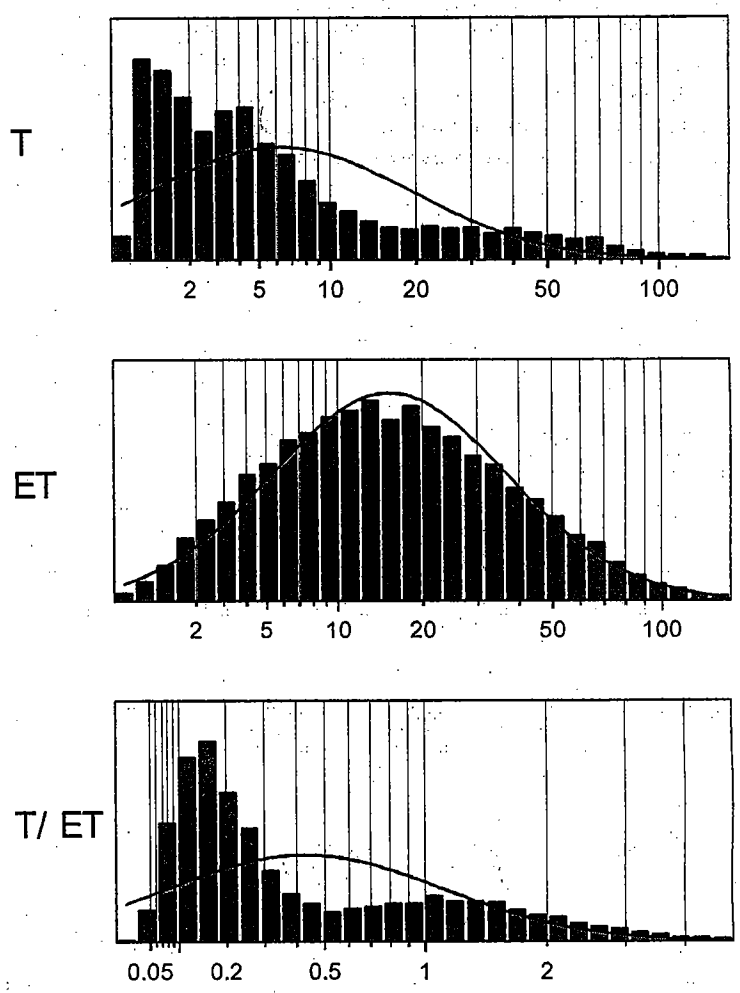
AHRENS
I believe it was the ^{13}C content of the plant, not the specifically of the sterols.

AUCHUS
What is the acetate? The acetate is going into the cholesterol that you are making. So again, the synthetic steroids are reflecting the sterol content unit which is not necessarily what is going into your body and then going into your cholesterol and then your sterol. I think that is the correct explanation, basically.

MATTHEWS
You are right about the acetate. You are what you eat. Same thing for these plants. They are what they fix. They are isotopicly light or heavy all the way through. So their acetate is isotopically light or heavy all the way through. The acetate may be a little lighter relatively to glucose, for example in these plants, but the overall plant is either isotopicly light or heavy.

This brings us back to the issue of corn. Because, I would suggest that if an Asian person who is not on a corn diet to begin with shifts his protein source around a little bit going to soy, there would almost be no change in the delta value of his steroids. Because it is probably the corn which is the most important aspect. It has nothing to do with the meat. It just has to do

Figure-1 Distributions of T parameters in international sports
(n=12,388: ng/ml)



Effect of High Soy Diet on GC-C-IRMS Results

Brian Ahrens
University of California, Los Angeles

Plants may be divided into two main types and several minor types. A C_3 plant takes up CO_2 and incorporates the C atoms into 3-phosphoglycerate or 3-Phosphoglyceric Acid (3-PGA). This is the so-called Calvin cycle of C_3 plants, of which sugar beet is an example. Glucose derived from C_3 plants has a $\delta^{13}C$ of about –25 $^{o}/_{oo}$ or lower. In contrast, when C_4 plants fix CO_2 the fixation yields a C_4-dicarboxylic acid (oxaloacetate). This process is also referred to as the Hatch-Slack cycle. The δ values found in C_4 plants range from about –8 to –20 $^{o}/_{oo}$ (heavier than C_3 plants because they contain more ^{13}C).

Starting materials for the synthesis of T are derived from the soy plant. Since soy plants are C_3 plants that are depleted in ^{13}C, we hypothesized that individuals who ingest soy diets will excrete urinary steroids with low ^{13}C values. To test the hypothesis we conducted a cross-sectional study consisting of 18 control subjects and 23 subjects living on a collective farm whose members were purported to subsist solely on soy products. The farm was located in Summertown, TN and the clinical aspects of the study were conducted at the farm and in the neighboring city of Knoxville, TN.

Compliance with the high soy diet was monitored by quantitating soy isoflavones, namely daidzein and genistein, in the urine of the subjects with an LC-MS-MS assay. The delta values of the acetates of A and E were determined by GC-C-IRMS.[1]

The median daidzein and genistein concentration in the urine of the control subjects was 205 and 213 ng/mL, respectively. In contrast the corresponding concentrations for the farm subjects were 1224 and 1966 ng/mL, respectively, thus indicating compliance with the soy diet.

The median $\delta^{13}C$ values for A acetates in the control group and farm group were –22.3 and –23.6 $^{o}/_{oo}$, respectively, and that this difference was significant by t-test ($p<0.001$). Similarly the median $\delta^{13}C$ values for E acetates in the control group and farm group were –23.7 and –24.9 $^{o}/_{oo}$, respectively, and this difference was significant by t-test ($p<0.001$).

Figure-2 Typical alteration of delta-values due to T doping

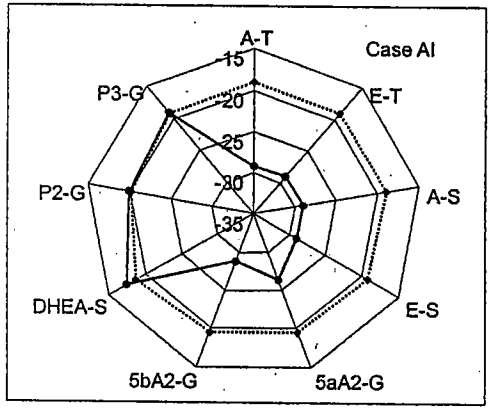


Figure-3 Delta parameters of steroids after DHEA application

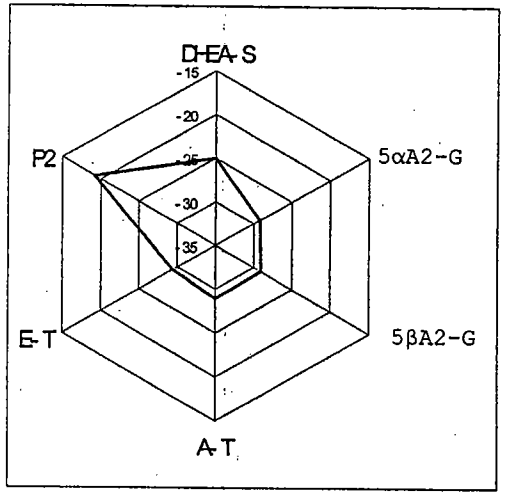
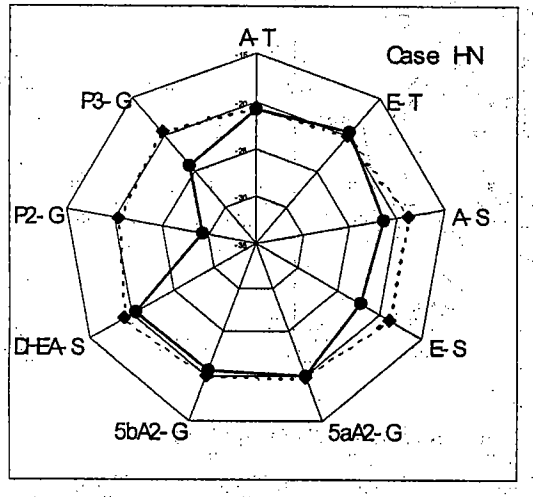


Figure-4 Alteration of delta-values of P2 and P3 after administration of pregnenolone



Question & Answer Discussion

WU
Do you use the oral administration?

UEKI
Yes, it is oral.

WU
Could you inform me of the trademark of the preparation? I know that in our laboratory, we only have the injectable preparation of the T propionate.

UEKI
T propionate is not available in our country. We administer a purified reagent for biochemistry. Why I chose propionate is because it is only effective orally.

SEGURA
Do you have an explanation, when you look at the natural values for your big range -14 to -22?

UEKI
Probably this high value was caused by the uncertainty of the measurement. What I wanted to show was the difference between these two compounds.

Effect of High Soy Diet on GC-C-IRMS Results

Brian Ahrens
University of California, Los Angeles

Plants may be divided into two main types and several minor types. A C_3 plant takes up CO_2 and incorporates the C atoms into 3-phosphoglycerate or 3-Phosphoglyceric Acid (3-PGA). This is the so-called Calvin cycle of C_3 plants, of which sugar beet is an example. Glucose derived from C_3 plants has a $\delta^{13}C$ of about –25 $^{o}/_{oo}$ or lower. In contrast, when C_4 plants fix CO_2 the fixation yields a C_4-dicarboxylic acid (oxaloacetate). This process is also referred to as the Hatch-Slack cycle. The δ values found in C_4 plants range from about –8 to –20 $^{o}/_{oo}$ (heavier than C_3 plants because they contain more ^{13}C).

Starting materials for the synthesis of **T** are derived from the soy plant. Since soy plants are C_3 plants that are depleted in ^{13}C, we hypothesized that individuals who ingest soy diets will excrete urinary steroids with low ^{13}C values. To test the hypothesis we conducted a cross-sectional study consisting of 18 control subjects and 23 subjects living on a collective farm whose members were purported to subsist solely on soy products. The farm was located in Summertown, TN and the clinical aspects of the study were conducted at the farm and in the neighboring city of Knoxville, TN.

Compliance with the high soy diet was monitored by quantitating soy isoflavones, namely daidzein and genistein, in the urine of the subjects with an LC-MS-MS assay. The delta values of the acetates of **A** and **E** were determined by GC-C-IRMS.[1]

The median daidzein and genistein concentration in the urine of the control subjects was 205 and 213 ng/mL, respectively. In contrast the corresponding concentrations for the farm subjects were 1224 and 1966 ng/mL, respectively, thus indicating compliance with the soy diet.

The median $\delta^{13}C$ values for **A** acetates in the control group and farm group were –22.3 and –23.6 $^{o}/_{oo}$, respectively, and that this difference was significant by t-test ($p<0.001$). Similarly the median $\delta^{13}C$ values for **E** acetates in the control group and farm group were –23.7 and –24.9 $^{o}/_{oo}$, respectively, and this difference was significant by t-test ($p<0.001$).

Figure-2 Typical alteration of delta-values due to **T** doping

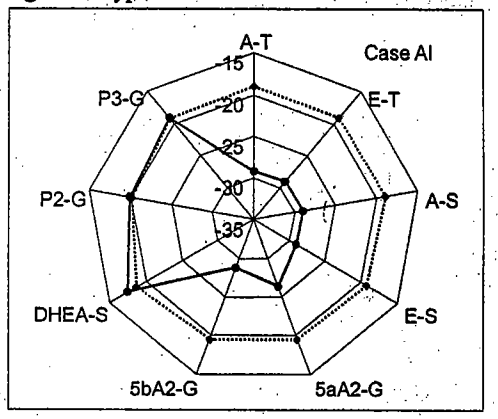


Figure-3 Delta parameters of steroids after DHEA application